

The effect of whole wheat and wheat milling fractions on metabolic parameters of adiposity, glucose control, and lipid metabolism using a rat model of obesity with type 2 diabetes

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Obesity prevalence is at an all-time high, with 1 in 3 Americans now classified as obese (1). Obesity is a debilitating condition that is associated with various co-morbidities, including type 2 diabetes (T2D), cardiovascular disease, pulmonary dysfunction, gastrointestinal disease, various cancers, and osteoarthritis. T2D is one of the most significant co-morbidities of obesity, as it often leads to other diseases such as non-alcoholic fatty liver disease (NAFLD) and hyperlipidemia.

There are now 347 million people worldwide with T2D (2). The incidence of newly diagnosed cases of T2D in America is at its highest of 1.9 million per year and, according to the American Diabetes Association, and if the trend continues, 1 in 3 Americans will have diabetes by the year 2050 (3). There are many factors that increase the risk of developing T2D, some which include being overweight and physically inactive, having a genetic predisposition, and high blood pressure. In addition, there are complications that develop gradually over a period of time during T2D if blood glucose sugar levels are not properly controlled. Long-term poor glucose control puts patients at a higher risk for complications such as cardiovascular disease (CVD), neuropathy, nephropathy, and retinopathy.

Obesity and diabetes are having a substantial impact on the economic state of the health care system. The total estimated cost of diagnosed diabetes in 2012 was \$245 billion. This includes \$176 billion in direct medical costs and \$69 billion in reduced productivity, encompassing lower labor force participation from chronic disability and premature mortality (4). In addition to the economic burden, there are substantial intangible costs, such as a reduced quality of life for those people with diabetes, and often for their families and friends as well.

Several epidemiological studies suggest that whole grain consumption is associated with a lower risk of obesity and T2D. Whole grain consumption has been found to have an inverse association with fat mass and insulin resistance in both humans and rats, possibly through several different mechanisms, involving mechanical, hormonal, and anti-inflammatory processes. The mechanical mechanism focuses on alterations in caloric density and absorption of nutrients, the hormonal mechanism on changes in hormone synthesis and secretion due to nutrient availability, and lastly, anti-inflammatory and anti-oxidative components in the whole grain. However, it is unclear which milling fraction of the whole grain, the bran, germ, or endosperm, may be responsible for these health benefits.

Therefore, the aim of this study was to examine the effects of whole wheat and wheat milling fractions, specifically bran, germ, and endosperm, on glucose control, insulin resistance, fatty liver, and adiposity using an animal model of obesity with T2D, the Zucker Diabetic Fatty (ZDF) rat. Male ZDF rats were fed either a cornstarch-based diet (AIN-93G; obese control), or diets containing 64% whole wheat flour, 54% refined wheat, 9.4 % wheat bran, 1.6% normal wheat germ, or high 15% wheat germ for 5 weeks. Lean ZDF littermates fed a standard AIN-93G diet served as a negative control. All animals were fed ad libitum. The refined wheat, wheat bran, and wheat germ were present in the diets in the same concentration as would be found in the whole wheat diet. The high wheat germ diet had amounts of germ 10 times that of the normal wheat germ diet. It was found that after 5 weeks, the whole wheat, refined wheat, and high and normal wheat germ groups all showed a significant improvement ($p < 0.05$) in area under the curve during glucose and insulin tolerance tests. There were no differences in body

weight or fat pad weight among the ZDF groups; however, there was a significant difference ($p=0.031$) in fat mass % between the whole wheat group and the obese control. The whole wheat group and all wheat fraction groups decreased the concentration of liver lipids compared to the obese control, and the bran and germ groups also had lower liver cholesterol concentration. Only the whole wheat group had a significantly lower cecum pH ($p<0.0001$) and greater cecal weight ($p<0.0001$) compared to the obese control, indicating greater fermentation of the diet. There were no significant differences in plasma adiponectin and resistin levels among the ZDF diet groups. In conclusion, the results of this study suggests that none of the wheat milling fractions stand out as responsible for the metabolic effects seen with consumption of whole wheat and that individual milling fractions of wheat are just as effective in improving insulin resistance and fatty liver as whole wheat.

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Chapter 1: Literature Review

Chronic Disease, Obesity, and Type 2 Diabetes

Obesity

Obesity is defined as having an excessive amount of adipose tissue, indicated by a body mass index (BMI) of 30 or higher, and is associated with an increased risk for numerous diseases and health complications, such as cardiovascular disease (CVD), type 2 diabetes (T2D), high blood pressure, stroke, cancer, sleep apnea, depression, gallbladder disease, infertility, skin problems, and delayed wound healing issues, all of which are significant health problems and reduce quality of life (1).

Obesity occurs when an individual consumes more energy than is expended through normal daily activities, exercise, and basal metabolic rate (BMR), causing the body to store the excess energy as fat. An individual's genetic background can also be a risk factor for obesity, as it may influence how the body responds to nutrients which may cause weight gain in some instances (2). Genetic and hormonal influences such as genetic mutations and hypothyroidism can affect body weight (3). There are also some more unusual causes that may be a result of underlying endocrine disorders. For example, Cushing's syndrome, polycystic ovary syndrome, and Prader-Willi syndrome all lead to decreased physical activity and therefore, weight gain (4, 5).

There are many approaches to treating obesity. Weight loss can generally be achieved with lifestyle changes such as increasing physical activity and altering eating behavior (6). Some of these diet modifications include following a low calorie diet, eating more fiber-rich foods such as whole grains, more plant based foods such as fruits and

vegetables, more lean sources of protein like beans, lentils, fish, lean beef, and meal replacements (7). Secondly, there are surgical methods that have been shown to be very effective at inducing weight loss such as gastric bypass and sleeve cut surgery. Lastly, pharmacological products such as Orlistat can assist in weight loss by inhibiting the absorption of fats from diet and therefore reducing the calories taken in by the body (8).

Type 2 Diabetes

T2D affects the body's ability to control blood glucose levels. The primary defect in T2D is the reduced effectiveness of the hormone insulin. Insulin is released from pancreatic β -cells into the bloodstream and targets the insulin receptor on muscle or adipose cells to cause the uptake of glucose (9). Several tissues show a reduced response to insulin and contribute to the development of T2D. The muscle and adipose tissue develop dysfunctional insulin signaling pathways, thereby preventing the uptake of glucose from the blood into the tissue cells (10). Additionally, the liver develops a selective insulin resistance that increases hepatic glucose output due to a failure in the glucose sensing mechanism (11). Without glucose uptake into the tissues, excessive glucose circulates in the blood stream and can glycosylate proteins, causing damage to the organs and nervous system (12).

When we consume a meal during normal, non-diabetic conditions, blood glucose levels rise and the pancreas is able to sense this through the glucose receptor GLUT2. β -cells in the pancreas then release insulin into the circulation, signaling the muscle and adipose tissue to absorb glucose from the blood via the GLUT4 receptor (13). When an individual is constantly eating foods high in glucose and/or highly processed foods, where starches

are rapidly digested into glucose, there is continually high blood glucose and blood lipids (pre-diabetic state) which with time, develops into T2D (14).

There are four stages to the progression of T2D. The first stage starts with normal insulin sensitivity but with high blood glucose levels, followed by the second stage, impaired glucose tolerance, which is due to a slight insulin resistance by insulin-sensitive tissues. As the cells become more insulin resistant, the third stage of hyperinsulinemia occurs, as the body tries to compensate for the insulin resistance by releasing more insulin into the blood. Eventually, in the last stage, there is an inadequate amount of insulin secreted (hypoinsulinemia) as the β -cells in the pancreas die due to lipid accumulation and oxidative stress (15).

Normal fasting blood glucose levels are typically below 100 mg/dL, while in a pre-diabetic state, blood glucose levels would range between 100 to 125 mg/dL. After an oral glucose tolerance test, if blood glucose remains lower than 140 mg/dL after 2 hours, then it is considered normal, but if it is between 140 to 199 mg/dL it is considered pre-diabetic, meaning impaired glucose tolerance. Higher than 200 mg/dL is considered diabetic (16).

There are various types of medications on the market to treat type 2 diabetes. These medications are divided into 4 groups as they work using different mechanisms. There are some that work by sensitizing the body to insulin (Biguanides and Thiazolidinediones), others that decrease hepatic glucose production (Dipeptidyl peptidase-4 (DPP4) inhibitors) (17), some that stimulate the pancreas to make more insulin (Meglitinides and Sulfonylureas) (18) and lastly, others that slow absorption of

starches from the intestine (Alpha-glucosidase inhibitors), which then slows the rate of glucose entering the blood stream (19).

Dietary and lifestyle changes are known to reduce the risk of T2D as well as maintain a healthy blood glucose level if already diagnosed with T2D. Some of these changes include exercise, consumption of monounsaturated and polyunsaturated fatty acids such as omega-3's, which can decrease free fatty acids (FFA) in the blood stream, a known contributor to inflammation and insulin resistance (20), consumption of fiber and whole grains, and more extreme dietary methods such as caloric restriction and invasive surgeries like gastric bypass (21-23).

Obesity related type 2 diabetes

During obesity, a risk factor for T2D as mentioned previously, there is a state of low-grade chronic inflammation which is thought to be a contributor to the progression of T2D. Excess white adipose tissue (WAT) can cause hypertrophy in the adipocytes leading to insulin resistance and increased adipogenesis (24, 25). This can result in an efflux of fatty acids from the adipocyte into the blood, which then circulate through the body and become deposited in the liver, muscle, and beta cells, causing disruptions in the insulin signaling pathway and therefore, cause insulin resistance (26-28). Increased amounts of WAT can also stimulate an immune response leading to release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α) and interleukin-6 (IL6) from the adipose tissue and from macrophages that have infiltrated into the adipose tissue from the blood, causing a low-grade inflammation (29). This inflammation also contributes to the insulin resistance already present in the liver and muscle, worsening

T2D. In addition to plasma FFA and macrophage-derived pro-inflammatory cytokines, the WAT also secretes the hormone leptin, which is involved with regulation of appetite and energy balance (30). During obesity leptin becomes ineffective as its receptors develop resistance due to the presence of saturated fats and ceramides that prevent its signaling from controlling appetite and body weight (31). Adipose tissue also secretes other adipokines, such as adiponectin and resistin, however when there is expanding WAT, the normal plasma concentration of these adipokines becomes altered (24). Adiponectin levels decrease with increased fat mass and insulin resistance (32). Adiponectin is known to improve insulin resistance by binding to the adiponectin receptors AdipoR1 and AdipoR2 and then activating adenosine monophosphate dependent kinase (AMPK) and PPAR- α (33). This activation then causes the cells in the liver and muscle to increase energy production pathways such as β -oxidation. Resistin on the other hand is correlated with increased body fat and is thought to increase insulin resistance (34, 35), but other studies have not found this (36). Lastly, during obesity, oxidative stress may increase as a result of an oversupply of FFA and glucose in the mitochondria which are then oxidized. As a result, there is greater reactive oxygen species (ROS) production which can activate protein kinase C (PKC) and c-Jun N-terminal kinase (JNK), leading to interruption of the insulin signaling pathway (37).

Insulin Resistance

Insulin resistance during T2D is defined as the impairment of glucose uptake into insulin sensitive tissues such as muscle, liver, and adipose tissue. It can lead to hyperinsulinemia as the β -cells in the pancreas secrete greater quantities of insulin to control the excess glucose in the blood (15). The main defect in insulin resistance in the muscle is an

impaired signaling pathway, caused by the inhibition of serine phosphorylation on IRS-1, prohibiting downstream signaling and in the end, preventing glucose uptake through GLUT4 (38). The inhibition of serine IRS-1 phosphorylation can be caused by inflammatory pathways, activated by the cytokine TNF- α , and also by the accumulation of intracellular lipids such as free fatty acids, diacylglycerol (DAG), and ceramides (39). These lipids, which act as signaling agents, are increased during obesity and T2D and have been shown to activate the JNK and I κ B kinase (IKK) stress response pathways to interrupt the insulin signal (40). Also, large amounts of ROS are produced through oxidation in the mitochondria due to the excess of fatty acids and glucose, which can cause mitochondrial dysfunction as well as mitochondrial death (41). Additionally, the liver becomes insulin resistant, and is no longer able to properly control glucose output while, at the same time, over-produces lipids from the excess glucose. Insulin resistance can also occur in the adipose tissue where it causes uncontrolled lipolysis, releasing more fatty acids into the circulation, further contributing to the accumulation of lipids in the liver and muscle (26-28). There are many mechanisms that cause insulin resistance but the focus here will be on three: inflammation, oxidative stress, and lipid accumulation in the liver.

Inflammation

A significant factor in the relationship between obesity and the development of insulin resistance is a chronic low-grade inflammation, particularly in the adipose tissue (42). During nutrient overload in adipose tissue, many stress pathways are triggered that stimulate the release of pro-inflammatory cytokines and adipokines into the circulation which contribute to the development of insulin resistance. Cytokines like TNF- α and IL-6

can act locally in the adipose tissue, initiating the recruitment of macrophages, which in turn secrete more cytokines and promote adipocyte cell death (43, 44). This creates a feed-forward loop that causes further inflammation. These pro-inflammatory cytokines circulate to other tissues, causing a constant, low-grade systemic inflammatory state (44, 45).

The low-grade chronic inflammatory state begins as a result of various effectors, some of which include endoplasmic reticulum (ER) stress, low adiponectin levels, higher leptin levels, uncontrolled lipolysis, macrophage infiltration, and adipocyte death (46). $\text{TNF-}\alpha$ is a pro-inflammatory cytokine produced mainly by macrophages and lymphocytes, however it can also be produced by adipose tissue in small amounts (47). $\text{TNF-}\alpha$ is thought to play an important role in insulin resistance through activation of stress kinases such as MAPK and JNK, which are able to phosphorylate serine residues on IRS-1, thereby stopping the insulin signaling pathway in the muscle (41). During obesity, the adipose tissue can over-express $\text{TNF-}\alpha$ and cause inflammation and a decrease in insulin sensitivity (46). $\text{TNF-}\alpha$ can also inhibit the nuclear receptor $\text{PPAR-}\gamma$, a transcriptional regulator of adipogenesis. As $\text{PPAR-}\gamma$ controls the transcription of genes involved in the synthesis of triglycerides, reduced activity results in greater free fatty acid release from the adipocytes into the circulation, which then build up in the muscle and liver to cause insulin resistance (48). Inflammation mediators such as C-reactive protein (CRP) are also released through the action of $\text{TNF-}\alpha$ by the liver (64).

IL-6 is a multifunctional inflammatory cytokine produced mainly by adipose tissue and is involved in the progression of insulin resistance in the liver and muscle tissue (49). It belongs to the cytokine class I receptor family that are responsible for activating the

JAK/STATs inflammatory pathways. IL-6 levels in the blood are increased during obesity and can directly affect lipid metabolism in the liver by inducing VLDL secretion and causing hypertriglyceridemia (46) resulting in further inflammation and insulin resistance. It is unclear as to how this happens but it has been suggested to involve either tyrosine phosphatase activation, which is necessary for proper insulin signaling, or interactions between suppressors of cytokine signaling proteins and the insulin receptor. However, not all findings support a role for IL-6 in promoting insulin resistance, as it has been reported by Nieto-Vazquez et al. (50) that mice treated with IL-6 for 3 h stimulated glucose uptake in skeletal muscle, suggesting that acute exposure to IL-6 can actually improve insulin resistance.

Altered secretion of adipokines

The secretion patterns of adipokines are also altered during obesity and inflammation, such that adiponectin decreases and resistin increases, further contributing to insulin resistance. Resistin is produced in small amounts by the adipose tissue while the majority is secreted by macrophages and monocytes (46). The function of resistin is currently not completely understood but it is thought that it can induce insulin resistance in the muscle and liver (34). In-vitro studies using adipocytes have shown that resistin reduces insulin-stimulated glucose transport as well as inhibits adipocyte differentiation, which suggests a link between obesity and insulin resistance within the adipose tissue (51). Obese mice, who are insulin resistant, have increased resistin levels (34). Further, it has been shown that infusion of resistin into lean animals induces insulin resistance (34). However, the relationship between resistin and insulin resistance remains uncertain, as it has been

reported that there is a decrease in the gene expression of resistin during insulin resistance (52).

Adiponectin is another adipokine that is highly expressed in adipose tissue and released in large amounts into the circulation (53). Plasma adiponectin levels are inversely associated with obesity and insulin resistance, with lower concentrations present in obese and diabetic individuals and greater concentrations in insulin-sensitive individuals, and with increases during weight loss and (54, 55). Administration of adiponectin has been shown to reverse insulin resistance caused by adiponectin deficiency (32, 56).

Adiponectin is believed to improve insulin resistance in the liver and muscle by activating AMP-activated protein kinase (AMPK), a kinase that acts as the master regulator for intracellular energy balance (57). Activated AMPK increases fatty acid β -oxidation in order to remove harmful intracellular lipids and promote insulin sensitivity in the muscle as well as decreasing intracellular malonyl CoA in the liver, which leads to lower rates of lipogenesis (58, 59). Adiponectin is also known to regulate liver glucose production by decreasing expression of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (60). In addition, it is also thought that adiponectin can reduce inflammatory responses caused by TNF- α and reduce the secretion of TNF- α from macrophages (61).

Oxidative Stress

Oxidative stress is a result of an imbalance between the production and disposal of ROS produced during energy metabolism. In the insulin resistant state, this imbalance is due to greater oxidation of fatty acids and glucose in the mitochondria (62-64). Some ROS produced include free radicals, such as superoxide ($\cdot\text{O}_2^-$), hydroxyl ($\cdot\text{OH}$), peroxy

($\cdot\text{RO}$), and hydroperoxyl ($\cdot\text{HRO}_2$), as well as non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) (65). These species can cause oxidation of proteins, lipids, and DNA and also inhibit enzymes in the electron transport chain, blocking mitochondria respiration and therefore diminishing normal cellular functions (41). All of these effects can cause cell injury and may lead to cell death. Increased ROS production and peroxidation of cellular components can lead to mitochondrial dysfunction and a reduction in mitochondria number (66). This dysfunction can then contribute to FFA and lipid accumulation, which may lead to the interruption of the insulin signaling pathway (67). Additionally, ROS themselves are known to inhibit the insulin signal by activating the PKC, JNK, and NF- κ B inflammatory pathways, as well as activating TNF- α and glucocorticoid signaling (64, 68).

Lipid Accumulation in the Liver (Nonalcoholic Fatty liver)

Non-alcoholic fatty liver disease (NAFLD) is a co-morbidity that accompanies T2D and obesity. This disease is classified as the accumulation of excess fat in the liver, accompanied by increased inflammation, which can eventually lead to more serious conditions such as liver scarring (69). Diagnosis of this disease requires evidence of hepatic steatosis, either by imaging or by histology, as well as another cause of secondary hepatic fat accumulation, hepatitis C infection, or medications (69). Diagnosis can be done by ultrasound imaging and measuring plasma levels of aminotransferase or, less commonly, by liver biopsy. World prevalence of NAFLD ranges from 6.3% to 33% in the general population whereas in obese and in T2D patients, the prevalence jumps to 90% and 69% respectively (70). NAFLD is associated with many metabolic risk factors

such as a high BMI, visceral obesity, diabetes mellitus, dyslipidemia, and hepatic carcinoma (69). The cause of NAFLD is believed to be due to an initial accumulation, due to increased influx from adipose tissue and increased conversion of glucose to fatty acids in the liver, of triglycerides in the liver, followed by increased oxidative stress in the hepatocytes, likely due to greater fatty acid β -oxidation (71). Sanyal et al. (71) found

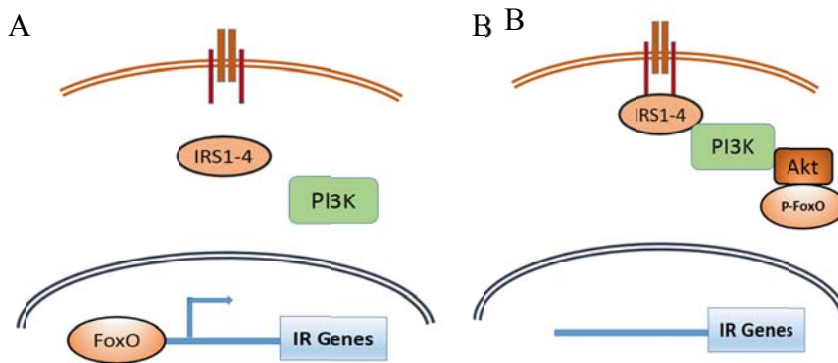


Figure 1-1. The regulation of gluconeogenesis phosphorylation of the FoxO transcription factor. Panel A shows transcriptional activation by FoxO of insulin-responsive (IR) genes, including those of gluconeogenesis. Panel B shows that insulin results in phosphorylation of FoxO, leading to a decreased transcription of IR genes. Redrawn from(73).

that patients with NAFLD had greater peripheral insulin resistance and increased plasma FFA derived from adipocytes. Increased plasma FFA, originating from uncontrolled lipolysis in insulin-resistant adipose tissue, are deposited in the liver and accumulate over time. NAFLD often occurs alongside hepatic insulin resistance, in which the liver goes through increased lipogenesis, generating an excess of triglycerides that remain in the

liver (11). The liver has selective insulin resistance during T2D in which there is increased glucose output through gluconeogenesis while, at the same time, greater fatty acid production from lipogenesis. In the normal situation, as shown in Figure 1-1, when insulin is low, the transcription factor FoxO binds to the promoter region for gluconeogenic genes, increasing their transcription and ultimately, increasing glucose production and secretion into the plasma. When insulin is in high concentration, such as after a meal, insulin can suppress gluconeogenesis by causing the phosphorylation of FoxO, which can no longer bind the promoter region (72). However, in the insulin resistant state, insulin is unable to cause the phosphorylation of FoxO, and inappropriately high rates of gluconeogenesis occur (73).

Additionally, the increase in insulin due to insulin resistance also activates the transcription factor SREBP-1c, which increases lipogenesis (11). These synthesized lipids either remain in the liver or are released into the blood within VLDL, as the liver cannot sense the high plasma glucose and attempts to send fat to other parts of the body to be used as energy (74).

This buildup of hepatic triglyceride can lead to a continuous β -oxidation of fatty acids in the liver, which can interfere with the normal function of mitochondria. This creates oxidative stress, which, when accompanied by adipose-derived pro-inflammatory cytokines such as TNF- α , is believed to play an important role in the progression of liver damage in NAFLD (75).

There are four stages to NAFLD (figure 1-2 (76)). The first stage is simple steatosis, second is non-alcoholic steatohepatitis (NASH), third is liver fibrosis, and the last stage is

cirrhosis (77). During the first stage of this disease, excess triglyceride accumulates in the hepatocytes, which is generally not considered harmful, but triggers the initial stages of inflammation. In the second stage, the development of NASH is characterized by greater inflammation and hepatocyte injury. In stage three, NASH develops into fibrosis, a persistent type of inflammation that results in the production of fibrous scar tissue around the hepatocytes and blood vessels. The last and most severe stage is liver cirrhosis where scar tissue develops from clumps of dead hepatocytes and debris from immune cells. This stage is irreversible and over time will cause liver failure (78). Treatment for this disease includes weight loss through exercise, an improved diet to reduce energy intake, and possibly an increase in consumption of bioactive compounds that may be beneficial in reducing oxidative stress (79).

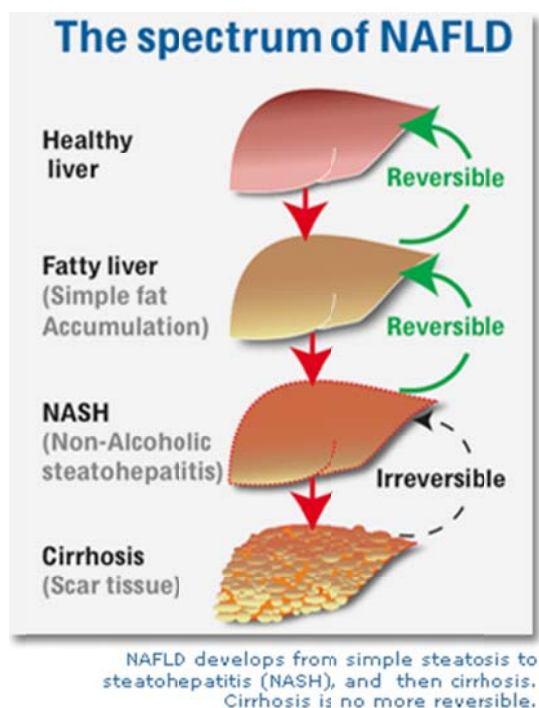


Figure 1-2. The stages of non-alcoholic fatty liver disease. I would like to acknowledge figure 1-2, as designed by the Kaori Minehira group

Whole Grains

There is growing evidence showing that whole grains play an important role in reducing the risk of chronic diseases like T2D and obesity, as well as reducing overall mortality rates, through improvements in glucose homeostasis (80, 81). Whole grains are composed of various vitamins, minerals, fiber, and bioactive phytochemical compounds which may have significant beneficial effects to health. Due to the large number of potentially beneficial components within whole grains, it is difficult to pin-point the actual component responsible, and therefore the mechanisms by which the whole grains may act to promote better health (80).

It has been reported that whole grain consumption is associated with a lower mortality rate relative to refined flours (82). As a result, there have been numerous studies conducted to examine the differences between diets high in whole grains and diets high in refined grains. With wheat being one of the most consumed grains in the United States, whole grain wheat has been widely used in research and is seen as a viable food production path for making changes that increase the overall health of the population. Observational studies (83-86) (87) and animal studies (88, 89) have demonstrated that whole wheat consumption is beneficial for reducing risk of obesity and T2D, but there is minimal research on identifying which component of whole wheat is responsible for the positive biological effects. Many researchers suggest that the health benefits could be due to the synergistic action of many compounds in the wheat (90, 91).

Wheat and Wheat Fractions

Wheat has become the predominate grain used in the United States food supply, as it has ideal properties for making consumer products such as breads, pastas, and baked goods. In addition, wheat is also consumed in side dishes and breakfast cereals as either wheat flakes or other forms like wheat berries (wheat kernels). There are two main varieties of wheat used in US food manufacturing, *Triticum turgidum durum* (durum wheat) and *Triticum aestivum vulgare* (bread wheat) (92). Bread wheat is described as soft or hard depending on what season the crop was harvested. Based on the color of the kernel, it is further classified as red or white (92). White wheat is most commonly used for making cookies, cakes, and pastries as it contains the least amount of gluten. It is also the most aesthetic in terms of color, has milder flavor characteristics, and less sweetener is needed to get the same level of sweetness than if red wheat was to be used. Red wheat has strong-flavored tannins compared to white wheat and has a higher percentage of gluten, and therefore, isn't widely used for products other than bread, making it somewhat less commonly consumed by the population. Hard wheat has more protein and is a winter crop grown in southern climates while soft wheat is a spring crop that is harvested in the fall (91). The wheat kernel is divided into 3 fractions, the bran, the endosperm and the germ, which can be separated by milling. These fractions are then sold and used for various food products such as bread making.

Whole wheat

A kernel of wheat is composed of roughly 80-85% endosperm (starch, i.e., refined wheat or white flour), 12-18% wheat bran and 2-3% wheat germ, each of which have different

macronutrient and micronutrient compositions that serve various functions for germination and growth of the plant (90). Due to the inclusion of germ and bran, whole wheat is considered to be more nutrient dense when consumed compared to the refined wheat fraction.

Bran

Wheat bran is a multilayer substance composed of various fibrous components: outer pericarp, inner pericarp, testa, nucellar epidermis, and the aleurone layer. Both pericarps are made up of branched heteroxylans, inulin, cellulose, and lignins that contain many cross-links between polymer chains due to the high content of ferulic acid dimers (80). The nucellar epidermis contains arabinoxylans, which contain the phenolic acids and other antioxidants. The aleurone layer makes up 50% of the bran fraction and is mainly composed of cell walls, which contain β -glucans (soluble fiber), some protein, arabinoxylans, cellulose (insoluble fiber), and ferulic acid. It is also rich in nutrients such as phenols, phytates, minerals, and B vitamins like niacin and folate.

Endosperm

Wheat endosperm is primarily composed of starchy carbohydrate and comprises 80-85% of the wheat kernel (80). Refined flour is predominantly wheat endosperm and during the milling process, the endosperm is separated from the bran and the germ, most of the nutrients are removed except for the carbohydrates. The endosperm contains starch, small amounts of proteins, iron, and various B vitamins and vitamin E. In the United States, almost all of the refined wheat flour is enriched to replace certain nutrients that are lost when the bran and germ are removed in the milling process (93).

Germ

The germ is 2.5-3.8% of the wheat kernel and is a potentially nutritious food supplement. It is a concentrated source of various healthy lipids, proteins, sugars, minerals, vitamins (especially tocopherols and B vitamins), phytosterols, policosanols, carotenoids, and flavonoids. The germ fraction contains relatively high amounts of unsaturated fatty acids and, because of this, can oxidize and become rancid quickly (94).

Whole Grains and Chronic Disease

Consumption of whole grain cereal products are associated with reduced obesity and lower insulin resistance, however the exact mechanism of action is unclear, as whole grains contain an enormous number of bioactive compounds that could be involved, such as phytochemicals, fiber, omega-3 fatty acids, trace elements, choline containing compounds, phytosterols, oligosaccharides, and B and E vitamins (90). Each of these compounds may have a beneficial impact on factors related to obesity and T2D, therefore it is difficult to pinpoint which individual components are responsible. As well as the individual compounds found in whole wheat, wheat is also separated into 3 different milling fractions, which can bring more uncertainty as to where the benefits are coming from. There have been few studies that compare the individual fractions of wheat using well-controlled designs, making it difficult to understand which milling fraction of whole wheat is most significant in lowering risk of chronic disease.

Epidemiological Studies

There have been a number of epidemiological studies that have reported an inverse association between whole grain intake, including wheat, and chronic diseases such as obesity and T2D. In a meta-analysis, consumption of high amounts of whole grain products, such as whole-wheat bread, were associated with a 20-30 % reduction in the risk of T2D (87).

In a prospective cohort study conducted in 2013, Parker et al. (83) found that whole grain consumption in postmenopausal women was associated with a decreased incidence of T2D. Women who consumed more than two servings per day of whole grains had a 43% reduction in the incidence of diabetes compared to women who did not eat any whole grains. Even those consuming only one serving per day showed a significant decrease in the hazard ratio for T2D incidence.

Another recent cohort study conducted by Wirstrom et al. (84) also reported an inverse relationship between high whole grain intake and the risk of diabetes. In this study, they investigated whether a higher intake of whole grain (59.1 g/d compared with 30.6 g/d) protected against the development of T2D in men and women 35-56 years of age. They found that higher whole grain intake (> 59.1g/d as opposed to 30.6 g/d) was associated with a 34% lower risk of T2D.

A prospective cohort study by Liu et al. (95) examined the associations of dietary fiber and whole or refined grain products with weight gain over 12 years, using food frequency questionnaires to collect dietary information. The results showed that, overall, women who consumed more whole grains weighed less (on average 1.5 kg) than women who

consumed fewer whole grains. The results suggest that with a dietary intake of 12 g of fiber there was less weight gain (8 lb) compared to those who had the smallest increase in intake, over the 12 year follow-up period. Weight gain was inversely associated with whole grain consumption but was also positively related to refined grain intake, suggesting that whole grains may be more beneficial for maintaining body weight than refined grains. In another study conducted by Steffen et al. (86), they observed that whole grain intake was associated with lower BMI and decreased insulin resistance in adolescents, especially among the heaviest persons.

Thus, epidemiology studies suggest that individuals who consume high amounts of whole grains have better weight management and glucose control than those consuming lesser amounts of whole grains and/or greater amounts of refined grains.

Animal studies

There have been a number of animal feeding trials investigating the effects of whole wheat, refined wheat, and wheat bran on T2D and obesity (88, 89, 96), however, there are relatively few studies on the effect of wheat germ.

In a study conducted by Adam et al. (88), the potency of different wheat milling fractions, whole wheat flour, white flour, and wheat bran, on lowering liver lipids and cholesterol as well as their effect on colonic fermentation, was evaluated in rats. The diet groups in this study consisted of each milling fraction. The whole wheat diet was composed of 70% whole wheat flour, the refined white flour diet was 48.6% refined flour and the wheat bran diet consisted of 21.4% wheat bran. The whole wheat-fed and bran-fed rats showed decreased hepatic cholesterol compared to the control group (fiber free)

and all wheat diets showed reduced liver triglycerides compared to the purified fiber-free control group. There were no differences in body weight after consuming the wheat diets for 3 weeks. Rats fed whole wheat flour and white flour showed a significant increase in cecum weight (+70% and +59%, respectively, $P < 0.001$) compared to control group, whereas rats fed the bran diet only showed a slight increase in cecum weight, suggesting greater fermentation in the 3 treatment diets. This study suggests that whole wheat flour, bran, and endosperm are all capable of reducing liver cholesterol and lipids, but have no effect on weight gain.

Another study conducted by Choct et al. (89) examined the effects of whole wheat and white (refined) flour from two wheat varieties that differed in fiber content on plasma cholesterol in rats. They also determined the concentration of soluble and insoluble fiber of the flours, which was different between the two wheat varieties, as this difference may result in altered digesta viscosity which may have an impact on cholesterol absorption. The flours were processed to a particle size of 2 mm and the diets were composed of 75% milled whole wheat and white (refined) flour and fed for 18 days. The results show that plasma cholesterol concentrations were lower in rats fed white wheat compared to whole wheat, regardless of wheat type, however there was no change in body weight. The amount of bile acids and cholesterol in the cecum was greater in rats fed the white wheat flours compared to the whole wheat flours but were unaffected by wheat variety. This study indicates that white wheat flour, in comparison to whole flour, may increase the excretion of cholesterol and bile acids in the feces, and that this may be the reason for the lower plasma cholesterol in these groups. The data from previously described animal studies are inconsistent, Adam et al. suggests that whole wheat, wheat bran and refined

wheat can lower liver cholesterol. However Choct et al. suggests that refined wheat lowers plasma cholesterol more than whole wheat and therefore reduces the risk of cardiovascular disease. These studies did not find an effect on body weight gain by refinement state of flour, and thus do not support the epidemiological associations of lower body weight with greater consumption of whole wheat.

Previously in our laboratory, we have shown that whole wheat consumption has a modest effect on the development of type 2 diabetes in the Goto–Kakisaki rat (97). Rats that were fed 65% whole wheat flour compared to a standard rat diet, the AIN-93G diet, had reduced fasting blood glucose after 2 months but not after 5 months. Whole wheat did not improve other measure of glucose control, including insulin secretion, glycated hemoglobin, oxidative stress, and β -cell mass. This study also examined the effects of other whole grains like oats, barley and maize, which also showed no effect on glucose control compared to the control diet. In this polygenetic model of diabetes without obesity, whole wheat consumption did not provide long term protection against the development of T2D.

Clinical studies

Clinical trials have shown that regular intake of whole grain cereals can contribute to lower risk factors related to non-communicable chronic diseases such as diabetes and obesity. In a short-term, randomized cross-over design study conducted by Kim et al. (98), obese women consumed a meal containing fiber from only wheat (primarily insoluble fiber), from only barley (soluble fiber) or a combination of the two grains. After a two day control diet, the women were given the test meal and their blood glucose and

insulin responses were measured for 3 h. There was no significant difference in the plasma glucose AUC, however the insulin response was significantly lower in the barley group compared to the meal containing only whole wheat. As a lower glucose and insulin response are beneficial for controlling blood glucose, this study suggests that whole wheat may not be helpful for reducing the risk of T2D compared to diets that have more soluble fiber. Clinical trials that fed whole grains for longer periods of time and measured variables more informative of insulin resistance, such as HOMA (an index of insulin resistance), or glycated hemoglobin, a measure of long-term blood glucose control, show no benefit compared to refined grains (99-101). In a crossover design study by Kristensen et al. (99), refined wheat was replaced by whole wheat in the diets of 79 overweight or obese women for 12 weeks. No differences were found in measures of blood glucose control, including fasting plasma glucose, plasma insulin, HOMA, or glycated hemoglobin. They also observed no changes in bodyweight or BMI, but did note a slight reduction in fat mass. In a randomized control trial, subjects were fed diets high in either refined wheat or whole wheat for 12 weeks (100). At the end of the study, there were no differences in fasting plasma glucose or insulin, HOMA, or another index of insulin resistance called QUICKI, between the refined wheat group and whole wheat group. In a smaller crossover design study, Giacco et al. (101) fed 15 subjects whole wheat foods or refined wheat foods for a period of 3 weeks. Here again, as in previous studies, fasting plasma glucose, insulin, and HOMA were not different between the groups. Plasma free fatty acids and bodyweight were also similar. Two other clinical trials using whole grain treatments also show no difference in bodyweight or BMI (102, 103). There have been few clinical trials that examine the effect of individual wheat milling fractions. Jenkins et

al. (104) examined the effect of wheat bran on glycemic control in a randomized crossover trial. After 3 months of consumption of 19 g/d wheat bran, there were no significant differences between the control and wheat bran groups in body weight, fasting blood glucose, or glycated hemoglobin. There is also little evidence that wheat germ is beneficial. In a 4 week study by Cara et al. (105), healthy adults were fed diets supplemented with 30 g of wheat germ per day. No changes in body weight or fasting plasma glucose were found, however they did observe a significant reduction in plasma triglycerides. Thus, the results of clinical trials do not appear to support the epidemiological observations suggesting that whole wheat consumption improves insulin resistance or reduces obesity.

Possible mechanisms of action of wheat on diabetes and obesity

While clinical studies show no effect of whole wheat on glucose control, epidemiological and some animal studies suggest otherwise, therefore possible mechanisms need to be investigated. Studies have been conducted to investigate mechanisms potentially responsible for the health benefits of whole wheat, particularly for the diabetic and obese populations. It is thought that the mechanism responsible is through decreasing blood glucose levels, liver lipids, and a decrease in body weight (106).

Several mechanisms by which blood glucose levels and cholesterol are reduced by whole wheat have been proposed, including properties of dietary fiber such as colonic fermentation and viscosity, a decreased rate of starch digestion due to slower gastric emptying, particle size effects, or the actions of phytochemicals. Colonic fermentation of dietary fiber by the gut microbiota leads to the production of short chain fatty acids

(SCFA) which may also have an impact on the body's metabolism by increasing transit time through the digestive tract and perhaps the rate of absorption of glucose. Increased viscosity of intestinal contents by dietary fibers slows the absorption of glucose, thus reducing the glycemic index of a meal (107). Reduced gastric emptying and an increased particle size of the diet slow the digestion rate of starch which can reduce the postprandial glucose curve. Lastly, phytochemicals such as ferulic acid, which is the predominant phenolic acid in wheat, may have anti-inflammatory and anti-oxidative properties which help decrease inflammation and oxidative stress and in turn, insulin resistance.

Dietary Fiber

Dietary fiber has consistently been shown to have health benefits such as decreased cholesterol. Wheat fiber is mainly composed of insoluble fiber that is minimally fermented, but it also has a small amount of soluble fiber in the form of arabinoxylans (108). The insoluble part primarily assists with bowel movements by increasing stool mass (109). Soluble fiber has several well accepted health benefits, such as lowering plasma cholesterol and improving blood glucose in type 2 diabetes patients, and may assist with weight maintenance and decreasing low-grade inflammation by decreasing plasma C-reactive protein levels (101). A high consumption of dietary fiber has also been associated with a lower risk of developing coronary artery disease, hypertension, obesity, T2D, and colon cancer (110).

To understand how dietary fiber may reduce plasma cholesterol it is necessary to understand cholesterol metabolism. Cholesterol is obtained either from endogenous production in the liver or from exogenous sources, that is dietary cholesterol. Cholesterol homeostasis is maintained by the liver and can be used for the synthesis of steroid hormones and integration into cell membranes (111). Excess cholesterol is stored as cholesteryl esters in the liver. The main route for cholesterol catabolism is through the synthesis of bile acids (111). Cholesterol in the body can be lowered through excretion of cholesterol itself or by bile acids in the feces. As bile acids are synthesized from cholesterol in the liver, greater excretion forces the liver to continuously synthesize new bile acids from stored cholesterol, thereby lowering circulating cholesterol (111).

Soluble fibers such as β -glucans have been shown to lower plasma LDL cholesterol. There are several possible mechanisms for this effect (112). One is through its effect on postprandial glycemia due to the viscosity of soluble fibers, which reduces the rate of intestinal absorption of glucose and in turn, decreases the level of insulin in the blood (113). Reduced insulin levels may lead to decreased cholesterol synthesis, as insulin may increase the gene expression of HMG-CoA reductase, the rate-limiting cholesterol synthesizing enzyme, through SREBP-1c (114). However, this mechanism seems unlikely, since viscous fibers have been shown to increase hepatic cholesterol synthesis in rats (115). Another possibility is that increased viscosity in the small intestine can hinder the emulsion and interfere with the formation of small micelles, limiting the ability of the digestive tract to absorb all the cholesterol present (116).

Particle size, starch structure, and the glycemic index

Starch structure in wheat can have a profound effect on the postprandial glucose and insulin responses by slowing the absorption of glucose (117). Diets that yield a reduced glucose and insulin response are believed to lower the risk of obesity and T2D (118). In a study conducted by Jarvi et al. (119), the effect of glycemic index in a diabetic population was examined by altering food structure but maintaining identical nutrient composition. The patients were given two meals that differed in the structure of the starch sources. One meal consisted of whole red kidney beans, parboiled rice and bread made from whole wheat grains while the other meal was sticky rice, ground kidney beans and bread made from ground wheat grains. The results showed that the AUC for blood glucose and plasma insulin were significantly lower ($P < 0.001$) in patients who consumed the meal that had a preserved structure and bigger food particle size. Similarly, Holt et al. (120) found that subjects fed coarse flour and cracked grain, which have a larger particle size and a less-disrupted starch structure, had decreased glucose and insulin responses compared to those fed fine flour. Contrarily, meals that contained white or finely ground whole wheat flours resulted in similar glucose responses. However, the standard whole wheat flour particle size may have been milled too finely for a difference to be discernable (121).

Further study of the influence of particle size was conducted by Vincent et al. (122), who examined the effect of fine and coarse bran (15 g/day) on gastric emptying and small bowel transit in a three way crossover study. This study included males and females of age 19-23 who were given a meal of either 15 g of fine or coarse bran, along with labeled rice, with gastrointestinal transit time measured using scintigraphic imaging. The results

showed that the time to reach 50% gastric emptying was significantly delayed by the coarse bran compared to the fine bran. However, there was no difference in small bowel transit time. The mechanism of action suggested was a reduced pyloric flow with the coarse bran meal due to an increased viscosity of the gastric contents. Another mechanism may be a selective retention of large particles (>2 mm) by the pylorus in the stomach, including particles containing starch, thus slowing movement of starch into the small intestine. Surprisingly, the addition of coarse bran or small plastic particles to the meal can actually speed up transit time in the small bowel, once the material has passed through the stomach (123). This may explain the laxation effect of bran.

The ratio of amylose to amylopectin in wheat starch can also have significant effects on the glucose and insulin responses to a meal (124). High amylose products produce a lower blood glucose and insulin response when compared with similar products high in amylopectin, due to reduced branching and the lower solubility of amylose (117). In-vitro digestion of chewed barley samples, differing in amylose content, showed that samples with a low amylose content were hydrolyzed more quickly than grains with a high amylose content (125). In a study conducted by Kabir et al. (124) the consumption of mung bean containing 35% amylose and cornstarch containing 0.5% amylose was evaluated in rats. They found that the high amylose mung bean diet group produced a lower glucose response compared to the low amylose cornstarch group. In another study in which rats were fed either a high amylose/low amylopectin starch or a low amylose/high amylopectin starch, the low amylose/high amylopectin group had a greater glucose and insulin response as well as a greater bodyweight and higher insulin resistance compared to the high amylose/low amylopectin (126). Therefore the presence of high

amylose rather than amylopectin appears to reduce the response of glucose and insulin to a meal.

Colonic fermentation

Certain types of indigestible carbohydrates consumed in a meal can be fermented in the colon by various bacteria (127). This fermentation results in the production of microbial metabolites including the SCFAs, acetate, propionate, and butyrate, as well as carbon dioxide and hydrogen gases, and vitamins K and B₁₂ (127). These microbiota-produced metabolites can interact with receptors on epithelial cells and sub-epithelial cells causing the release of hormones that affect food intake and energy metabolism of the host (127). There have now been several studies demonstrating that whole grains are able to change the gut microbiota composition in the colon, increasing species that produce SCFAs (128). It has been demonstrated that the presence of SCFA in the colon can promote the secretion of PYY and GLP-1, which are able to slow the rate of gastric emptying and small bowel movement (129). However, recent evidence suggests that only fermentation of fructans such as inulin, is able to increase the concentration of PYY in the blood compared to other fermentable fibers like β -glucans (130). Soluble fibers such as guar gum, inulin, and pectin are highly fermentable while insoluble fibers like cellulose and wheat bran are minimally fermented (131).

Cani et al. (132) has shown that supplementation of the fructans oligofructose, a short chain fermentable fiber, in the diet of high fat-fed diabetic mice for 4 weeks, improved their glucose tolerance, fasting blood glucose, and glucose-stimulated insulin secretion, in addition to reduced body weight gain. However, in a long-term study conducted by Isken

et al. (133), mice fed the soluble fiber guar gum for 45 weeks showed a significant increase in body weight as well as increased plasma glucose AUC during an insulin tolerance test compared to mice fed insoluble fiber. It was suggested that soluble fiber increased energy extraction from the diet, causing weight gain. Lastly, in a human study by Luo et al. (134), type 2 diabetic subjects who consumed 20 g/d of short-chain fructooligosaccharides for 4 weeks had no improvement in their fasting plasma glucose, plasma glucose AUC during an insulin tolerance test, or glycated hemoglobin compared to a control group who were fed sucrose. At this time the data suggest that fermentation of soluble dietary fibers may not be able to reduce the risk of T2D.

Ferulic acid in the prevention of diabetes

Ferulic acid (FA) is a phenolic acid found in the seeds and leaves of most plants, and is the predominant phenolic acid found in wheat bran (135). FA is an antioxidant that neutralizes free radicals such as superoxide, nitric oxide, and hydroxyl radicals that may cause oxidative damage to cell membranes and DNA (136, 137). Whole grains such as wheat and oats have a high concentration of ferulic acid. However, the ferulic acid is mostly bound to the cell wall in the grains and therefore is only slightly bioavailable due to limited release by gut microbiota (138). However, ferulic acid can be freed from cell wall components through processing methods such as fermentation and alkaline treatment. Ferulic acid supplementation at relatively low doses has been shown to increase the activities of antioxidant enzymes, thus neutralizing free radicals which, in diabetes, are a primary cause of accelerated tissue damage (139). While ferulic acid is a strong antioxidant, it is unlikely that the amount absorbed from wheat, where it is bound and unavailable, has a meaningful effect on overall antioxidant status through classical

mechanisms as a free radical scavenger (139). However, supplementation of ferulic acid has beneficial effects on various chronic diseases, especially type 2 diabetes (135, 140). In streptozotocin-induced diabetic rats, supplementation of purified ferulic acid (10 mg/kg/d) for 6 weeks resulted in lower fasting blood glucose, TBARS, and free fatty acids and greater pancreatic islet mass (141). In another study using OLETF rats, a less severe model of insulin resistance than streptozotocin-induced diabetes, ferulic acid (10 mg/kg/d) supplemented for 20 weeks resulted in a decrease in the plasma glucose AUC during glucose and insulin tolerance tests and greater plasma adiponectin, compared to rats not supplemented with ferulic acid (135). In high fat-fed mice, ferulic acid supplementation for 7 weeks decreased body weight and plasma and liver triglycerides (140). These studies suggest that supplementation of ferulic acid may be a viable therapeutic method for reducing the progression of obesity and T2D.

Phytosterols as a mechanism in decreasing cholesterol

Studies have shown that plant sterols are able to decrease intestinal cholesterol absorption and therefore reduce LDL cholesterol in the plasma (142, 143). Plant sterols are similar in structure to cholesterol and therefore use the same mechanism for solubilization in the lumen and for absorption from the intestine lumen into the enterocytes. Plant sterols are more hydrophobic compared to the cholesterol and therefore have a higher affinity for incorporation into the micelles. This causes the cholesterol to remain in the lumen instead of the micelles, therefore reducing intestinal absorption and increasing cholesterol excretion in the feces (144). The presence of plant sterols in the wheat germ could therefore have an impact in lowering LDL cholesterol.

Study Aim

Evidence from epidemiological and animal studies suggests that whole grain intake is beneficial in decreasing obesity and insulin resistance, whereas clinical studies do not show the same result. Further work is necessary in this area to more fully understand the underlying health benefits of whole grains, particularly whole wheat. Currently there are no studies that investigate the effect of individual wheat milling fractions, compared to whole wheat, on T2D and obesity. Therefore, the aim of the following study was to examine the effects of whole wheat and wheat milling fractions on glucose control, insulin resistance, and fatty liver using an animal model of obesity with type 2 diabetes.

Chapter 2: Whole Wheat and Wheat Milling Fractions

Improve Glucose Control and Reduce Non-Alcoholic Fatty

Liver in the Zucker Diabetic Fatty Rat

Purpose: Whole wheat has been shown to improve glucose control and insulin resistance, and decrease fatty liver. However, which milling fraction is responsible for these positive effects is unknown. Therefore, this study examined the effects of whole wheat flour and its milling fractions, bran, germ and endosperm (refined wheat), on glucose control, insulin resistance, fatty liver, and adiposity, in an animal model of obesity with type 2 diabetes, the Zucker Diabetic Fatty (ZDF) rat.

Methods: Male ZDF rats were fed ad libitum either 64% whole wheat flour, 54% refined wheat, 9.4 % wheat bran, 1.6% wheat germ or 15% wheat germ (hi-germ) for five weeks. ZDF obese control and lean ZDF littermates (negative control) groups were fed a purified diet containing 50% cornstarch. The refined wheat, wheat bran and wheat germ diets were present in the same concentration as would be found in the whole wheat diet.

Results: After 5 weeks, the whole wheat, refined wheat, wheat germ, and high-germ groups showed significant reductions in plasma glucose AUC during glucose and insulin tolerance tests compared to the obese control ($p < 0.05$). There were no differences in body weight or fat pad weight among the ZDF groups but there was a significant reduction in fat mass % in whole wheat group compared to the obese control group ($p = 0.031$). The whole wheat group and all wheat fraction groups decreased the concentration of liver lipids compared to the obese control and the bran and both germ groups had lower liver cholesterol ($p < 0.05$). Only the whole wheat group had both a greater cecum pH ($p < 0.0001$) and cecal weight ($p < 0.0001$) compared to the obese control, indicating greater fermentation of whole wheat diet. There were no significant differences in plasma resistin concentrations among the ZDF diet groups. However, plasma adiponectin

concentrations were significantly lower in the whole wheat group compared to the obese control group ($p= 0.032$).

Conclusion: The results show that each individual milling fraction is as beneficial as whole wheat itself in slowing development of insulin resistance and reducing fatty liver, and that the effects of the milling fractions are not additive.

Introduction

Chronic non-transmittable diseases (NCD) such as obesity and type 2 diabetes (T2D) previously occurred predominantly in developed nations but are now becoming more common in developing countries, outnumbering transmissible diseases (3). There are now 347 million people worldwide with T2D. In the United States alone there are 18.8 million people with T2D and another 79 million people in a pre-diabetic state (3). The incidence of newly diagnosed cases of diabetes is at its highest with 1.9 million per year. It is predicted that 1 in 3 Americans will have diabetes by the year 2050 if these trends continue (145). Often accompanying T2D, obesity is also at an all-time high with 1 in 3 Americans being obese (1). Due to the magnitude of the prevalence of obesity and T2D, they are now in the top ten causes of death in North America, and therefore are among the greatest public health concerns. (1).

Changes in lifestyle, food habits, and decreased physical activity are implicated in the rapid increase in obesity and T2D. The combination of diabetes and obesity is particularly harmful as it increases the risk of comorbidities, such as heart disease, kidney disease, nerve damage, dyslipidemia, blindness, and nonalcoholic fatty liver disease (NAFLD), drastically increasing mortality rates. Occurring in 70% of type 2 diabetes patients, NAFLD results from increased fat accumulation in liver cells (146). This can cause inflammation and increase the rate of gluconeogenesis which will aggravate hyperglycemia and increase the risk of T2D (147).

Several epidemiological studies suggesting that whole grain consumption can decrease the risk of obesity and T2D. A recent observational cohort study found that people who ingested whole grain cereals had 30% less risk of developing type 2 diabetes (84). Whole grain consumption has been found to be inversely associated with body fat mass and insulin resistance in both humans and rats (83). However, it is unclear which milling fraction of the whole grain, the bran, germ, or endosperm, is responsible for these health benefits. The bran contains high concentrations of dietary fiber, which is thought to reduce obesity and diabetes by slowing down the digestion and absorption of digestible carbohydrates as well as increasing feelings of fullness (148). It also contains large quantities of ferulic acid which has been suggested to have anti-inflammatory and hepatoprotective properties and to improve glucose control (135, 140). The germ is high in vitamins, minerals and essential fatty acids which may reduce inflammation and improve immune system function (149). Currently there are no studies that investigate the effect of individual wheat milling fractions, compared to whole wheat, on T2D and obesity. Therefore, the aim of this study was to examine the effects of whole wheat and wheat milling fractions on glucose control, insulin resistance, and fatty liver using an animal model of obesity with type 2 diabetes.

Methods and Materials

Animal Model

Choosing the correct animal model for the research objective is very important, as it will dictate the reliability of the results. The Zucker Diabetic Fatty (ZDF) rat was chosen in this experiment because it is a good model for studying the initial stages of type 2

diabetes with obesity. This rat model has a nonsense mutation in the leptin receptor gene, which is normally expressed most abundantly in the brain (150). This results in a defective leptin receptor, resulting in an absence of leptin signaling in the brain and causing hyperphagia. Characteristics of this model include obesity, insulin resistance, hyperinsulinemia, type 2 diabetes, hyperlipidemia, fasting hyperglycemia and hypercholesterolemia (151). At 5 weeks of age their initial weight ranges from 80 to 140 g and at week 10, from 280-380 g (152). The ZDF rats have lean ZDF counterparts which are much smaller and do not develop obesity or T2D. Since our research objective was to assess metabolic changes associated with the onset of obesity and T2D during a whole wheat and wheat milling fraction dietary intervention, the ZDF rat appeared to be an appropriate model.

Housing and care

The ZDF rats were purchased from Charles River Laboratories (Wilmington, MA) at 5 weeks of age. They were housed individually with a 12h light and dark cycle with food and water provided ad libitum. The animals were handled according to the National Institutes of Health guidelines and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee.

Diet

There were 7 diet groups of 10 animals each. The positive control (ZDF) and the negative control (ZDF lean) were fed the AIN-93G purified diet (153). The remaining 5 ZDF

groups were fed diets containing either 64% whole wheat flour, 54% refined wheat, 9.4% wheat bran, 1.6% wheat germ, or 15% wheat germ for 5 weeks (Table 1).

Soft white wheat and its milling fractions were sourced from a milling company (Star Of the West Milling Co., Frankenmuth, MI) and stored at 4° C. A proximate analysis was conducted to determine the protein, carbohydrate, lipid, total dietary fiber, and ash content of the whole wheat and milling fractions (Medallion Laboratories, MN, USA). The results of the analysis were used to formulate the diets, ensuring that they were matched for macronutrient content and therefore were isoenergetic. A final analysis of the diets was conducted to check for the concentration of resistant starch and fibers. The diets were formulated so that the amount of the milling fraction present in the bran, refined, normal wheat germ diets were the same as they would be present in the whole wheat diet. The high wheat germ diet had 10 times the amount of germ present in the normal germ diet.

Experimental design

The animals were fed the diets ad libitum for 5 weeks. A meal tolerance test was conducted at the end of week 1, an insulin tolerance test and a glucose tolerance test at the end of week 3, a urine collection at the end of week 4, and a 48 h fecal collection was done at the beginning of week 5. In addition, body composition was determined by dual x-ray absorptiometry (DXA) scanning (Lunar Prodigy Advance, GE Healthcare, Piscataway, NJ) at the end of week 4. At the end of week 5, 12 h fasted rats were anesthetized by isoflurane, blood collected by cardiac puncture into tubes containing EDTA (1 mg/mL), and the kidneys, liver, epididymal, inguinal, and retroperitoneal fat

pads were harvested and stored at -80° C. The blood was centrifuged, plasma collected, and then stored at -80° C until analysis. The pH of the cecal contents was measured using a combination spear-tip pH electrode (model 81-63, Orion Research, Boston, MA) and then the cecum was emptied of contents, flushed with distilled water and weighed. Feces were freeze-dried, weighed and stored at -50 °C. At 4 weeks, rats were placed in metabolic cages with free access to food and urine collected over 24 h which was then frozen at -50 °C until analysis. Body weight was measured weekly and food intake was measured at weeks 1, 2, and 3.

Plasma analysis

Plasma glucose was measured in whole blood using a glucometer (AlphaTrak, Abbott Laboratories, Abbott Park, IL) calibrated for rodents. Fasting plasma adiponectin was measured using an ELISA kit (Millipore, Billerica, MA) and fasting plasma resistin was measured using an EIA kit (Cayman Chemicals, Ann Arbor, MI). Fed-state plasma insulin was measured using a rat-specific radioimmunoassay kit (Millipore).

Meal, glucose, and insulin tolerance tests

During the meal tolerance test, 12 h fasted rats were fed 2.5 g (1.33 g digestible carbohydrate) of their respective diets and allowed 20 minutes to consume the meal. Blood glucose was measured at time intervals of 0, 15, 30, 60, 90, and 120 minutes after presentation of the meal and ~250 µL blood collected at time points 0, 30, 60, 90, 120, and 180 minutes to measure plasma insulin.

During the glucose tolerance test, 12 h fasted rats were given glucose via gavage (0.5 g/kg) and blood glucose was measured using a glucometer at time points 0, 15, 30, 60, 90, 120, and 180 minutes. Area under the curve (AUC) was calculated by the trapezoidal rule, using a blood glucose concentration of zero as a baseline.

During the insulin tolerance test, insulin (1 IU/kg) was injected into the abdominal cavity and blood glucose was measured at time points 0, 15, 30, 60, 90, 120, and 180 minutes and AUC calculated as above.

TBARS and urinary ferulic acid analysis

Urinary thiobarbituric acid-reactive substances (TBARS) were measured using a previously described method (154).

Urinary ferulic acid was measured by HPLC after deconjugation using a modification of the method of Bunzel et al. (155) Briefly, ferulic acid was deconjugated by mixing 5% of 24 h collected rat urine with the same volume of 1 M sodium acetate buffer (pH 4.9), 300 μ L of sulfatase solution (Sigma, S9626-10KU, ST. Louis, MO), and 100 μ L of 0.1 mg/mL alpha coumaric acid solution (in 50% aqueous methanol). The mixture was incubated 37 °C for 3 h. The reaction was stopped with concentrated hydrochloric acid 0.3 mL and ferulic acid extracted with a total of 4 mL ethyl acetate (2 mL, 1 mL, 1 mL). The pooled ethyl acetate phases were extracted with 5 mL of 5% sodium hydrogen carbonate (2 mL, 2 x 21.5 mL) and phases separated by centrifugation. The combined sodium hydrogen carbonate phases were acidified with 0.5 mL of concentrated hydrochloric acid until the pH was <2, and then extracted with 3 x 2.5 mL ethyl acetate.

The ethyl acetate phases were collected and dried under nitrogen. The dried extract was reconstituted in 200 μ L 50% methanol and 1:5 diluted for HPLC analysis.

Quantification of ferulic acid was achieved using a Shimadzu HPLC system with auto sampler and SPD-M20A PDA detector. HPLC conditions are as follows: A Luna Phenyl-Hexyl column (250 x10 mm; 4.6 μ m particle size) was used for separation at a flow rate of 1 mL/min and temperature of 45° C. Mobile phases were 0.1 mM trifluoroacetic acid (TFA) (mobile phase A), 90% methanol/10% TFA (mobile phase B) and 90% acetonitrile/10% TFA (mobile phase C). The following gradient program was used (156): 87% A and 13% C for 10 min; change over 10 min to 77% A, 3% B, and 20% C; change over 5 min to 70% A, 5% B, and 25% C; and finally change over 5 min to 25% A, 25% B, and 50% C. Detection wavelengths were 280 and 325 nm.

Liver lipids and cholesterol

Liver lipids were extracted with organic solvents using the method of Fölch et al. (157). The solvent was evaporated under nitrogen, and lipids determined gravimetrically. Liver cholesterol concentrations were determined enzymatically as previously described by Gallaher et al. (158) and fecal bile acids extracted and purified by the method of Locket and Gallaher (159) and quantified using the method of Sheltawy (160).

Statistical analysis

The results were analyzed by ANOVA using the SAS statistical program (SAS 9.3, SAS Institute, Cary, NC). The group means were compared by Duncan's multiple range test

and comparison of fat mass % between the obese control and whole wheat groups was done using Student's t-test. Final body weight of the obese control and whole wheat groups was compared using Student's t-test. Statistical significance was taken at $p < 0.05$.

Results

Whole wheat and milling fractions do not alter body weight and food intake

At the end of 4 weeks, the ZDF groups had significantly greater body weight compared to the lean group, as expected due to the animal model. However, there were no significant differences in weight gain among the ZDF groups (Table 2). Additionally, while the ZDF groups did have a greater mean food intake than the lean group, there were no significant differences in food intake among the ZDF groups (Table 2).

Whole wheat produces greater fermentation

Rats fed the whole wheat diet showed a significant decrease in cecum pH compared to the obese control, suggesting greater fermentation of this diet (Table 2). The cecum weight was significantly greater in the wheat based diets compared to the obese control except for the wheat bran group (Table 2). Among the wheat diet groups, the whole wheat showed a significantly greater cecum weight compared to the other wheat diets, further suggesting greater fermentation in this group.

Whole wheat, but not milling fractions, reduce fat mass %

The obese control had greater epididymal, inguinal, and retroperitoneal plus perirenal fat pad weights compared to the lean group, as expected, but there were no significant differences among the ZDF groups in fat pad weights (Table 2).

Likewise, a DEXA scan did not show significant differences in fat mass or lean mass percentage among the ZDF groups (Table 2). However, when compared by Student's t-test, the whole wheat group showed a significant decrease in fat mass percentage compared to the obese control ($p=0.025$).

Whole wheat and wheat milling fractions reduce insulin resistance.

A meal tolerance test was conducted at week 1 to examine the effect of the diets on the uptake of glucose and the insulin response to the diet. There were no differences in the plasma glucose AUC during the meal tolerance test between the obese control and the wheat milling fraction diet groups, although wheat bran did have a significantly greater AUC compared to the high wheat germ (Table 2). The obese control group had a significantly greater insulin AUC than the lean control but there were no differences among the ZDF groups in the insulin AUC.

After 3 weeks on the experimental diets, the fasting plasma glucose concentration of the whole wheat diet and refined wheat groups was significantly less compared to the obese control group while wheat bran, wheat germ, and high wheat germ were not different (Table 2).

During the GTT at week 3, all wheat diets, except for wheat bran, had a reduced plasma glucose AUC compared to the obese control group (Table 2). Similarly, during an ITT,

also performed at week 3, the plasma glucose AUC was significantly lower in all ZDF wheat diet groups compared to the obese control (Table 2).

A greater kidney weight as a percentage of body weight is considered a marker of poor blood glucose control (161). The kidney weight percentage was lower in the lean group compared to the ZDF groups, but there were no differences among the ZDF rats (Table 2).

Urinary 24 h TBARS was measured as a marker of whole body lipid peroxidation (Table 2). Urinary TBARS was significantly greater in the obese control group compared to the lean control group. Urinary TBARS was significantly greater in the whole wheat, refined wheat, wheat bran, and high wheat germ groups than the obese control group, and whole wheat was greater than all other groups except high wheat germ.

Plasma adiponectin, an adipokine that is generally inversely proportional to body fat and insulin resistance, was in fact greater in the obese control compared to the lean group (Table 2). The whole wheat group had a significantly lower plasma adiponectin concentration compared to the obese control whereas the individual wheat milling fraction groups were not different from the obese control group. Resistin, another adipokine, is known to increase in models of obesity and T2D and has been suggested to be a link between obesity and insulin resistance (162). Plasma resistin was greater in the obese control group compared to the lean group. Although there were no differences among the ZDF groups, plasma resistin in the whole wheat and wheat milling fraction groups did not differ from the lean group (Table 2).

Fatty liver is reduced with whole wheat and wheat milling fractions.

The liver weights of the lean group were significantly less than the ZDF groups, yet there were no differences among the ZDF groups (Table 3). The whole wheat and wheat fraction diets, however, had lower total liver lipids compared to the obese control.

Total liver cholesterol concentration was significantly lowered in the wheat bran, germ, and high germ groups compared to the obese control, whereas the whole wheat and the refined wheat only showed a trend in decrease (Table 3). Liver free cholesterol concentration was not different among the ZDF groups. There was no difference in bile acid excretion between the obese control and lean groups, yet among the wheat groups the whole wheat and wheat bran groups had significantly greater fecal bile acid excretion compared to both the lean and obese control groups (Table 3). Fecal bile acid excretion did not correlate with liver cholesterol concentration ($r^2=0.05$, $p = 0.67$).

Whole wheat and wheat germ groups had the greatest absorption of ferulic acid

Ferulic acid, a phenolic acid found in wheat, has been suggested to slow the progression of diabetic complications (135, 163). The whole wheat group had the greatest intake of total ferulic acid followed by wheat bran, high wheat germ, refined wheat and, lastly, wheat germ (Figure 1). As there were differences in the relative concentration of total and free ferulic acid in the wheat diets, the daily intake of free ferulic acid was slightly different. The refined wheat group had the greatest free ferulic acid intake, followed by whole wheat, high wheat germ, wheat bran, and wheat germ (Figure 1). Urinary excretion of ferulic acid, a measure of ferulic acid absorption, mirrored the concentration present in the individual diets, with whole wheat having the greatest urinary ferulic acid excretion, followed by bran, high wheat germ, refined wheat and, lastly, wheat germ.

Discussion

There is considerable epidemiological evidence indicating that consumption of whole grains, of two or three servings per day, in comparison to more refined grains, are associated with a lower risk of obesity (164, 165) (decrease in body weight and a change in body composition) and type 2 diabetes (85, 149), although there are also studies that show no effect (166). When wheat is milled, the bran and germ are removed, and most of the dietary fiber, vitamins, minerals, essential fatty acids, and phytochemicals are lost. It is unknown which milling fraction of the whole wheat, the bran or the germ, may be responsible for the putative health benefits. Therefore, we investigated the effect of whole wheat and its individual milling fractions, endosperm, bran, and germ, on parameters of glucose control, insulin resistance, adiposity, and fatty liver using an animal model of the initial stages of diabetes with obesity, the ZDF rat.

After 5 weeks consuming the diets, there were no differences in body weight or fat pad weights among the ZDF groups, suggesting that neither whole wheat nor its bran or germ milling fractions were more effective than endosperm (refined wheat) at minimizing weight gain and body fat. Although whole grain intake is highly associated with a lower body weight in observational studies (95, 167), a direct relationship is not supported in clinical trials (102, 103). In the present study, no decrease in body weight was observed with consumption of whole wheat, yet a decrease in body fat percentage in the whole wheat group compared to the obese control was found by DEXA scanning. These findings are similar to a study conducted by Kristensen et al. (99), in which overweight or obese women fed isoenergetic diets containing either refined or whole wheat for 12

weeks showed no difference in body weight but did have a decrease in body fat percentage by DEXA, implying a change in body composition. A study in women conducted over 20 months reported that for every 1g increase in total fiber consumed a day body weight decreased by 0.25 kg and body fat by 0.25% (168). However, in the present study all diets were isofibrous, so changes in dietary fiber consumption does not explain the reduction in body fat experienced by the whole wheat group, as measured by DEXA scanning. A possible reason as to why no differences in the fat pad weights were seen could be the greater variability in fat pad weights compared to DEXA scanning, as a result of the challenge in precise removal of fat pads in these extremely obese animals. There were no differences in body weight or body fat percentage with the milling fractions compared to the obese control. This is consistent with other studies reporting that wheat bran fed to obese Zucker rats did not alter either final body weight or fat pad weight (169). Although glucose intolerant subjects given wheat bran as a supplement to their diet experienced a slight but statistically significant decrease in body weight (170), body weight in patients with diverticular disease experienced no change in weight with wheat bran consumption (171).

A possible explanation for the reduced fat mass percentage in the whole wheat group could be the effect of fermentation. After 5 weeks on the diets, the whole wheat diet group showed a significant decrease in pH level and greater cecum weight, suggesting greater fermentation of the diet. In a similar study pigs were fed different whole grains, including whole wheat, and saw an increase in fermentation which was believed to be due to fermentation of the soluble fiber arabinoxylans present in cereals (172). Adam et al. (88) also saw decreased pH and increased cecum weight in rats fed whole and refined

wheat, but not with wheat bran, which can be attributable to the small amounts of soluble fiber present in bran. Fermentation increases production of short chain fatty acids (SCFA), which have been shown to increase the release of the satiety peptides PYY and GLP-1 in some cases (173) but not others (130), depending on the type of fermentable fiber.

The reduction in fasting blood glucose and glucose AUC during an ITT and GTT suggests that all wheat diets led to a significant improvement in glucose control and reduction in insulin resistance compared to the obese control group. However, there was only a trend for a lower GTT AUC and fasting blood glucose in the wheat bran group compared to the obese control group. A decrease in kidney weight as a percentage of body weight is considered a secondary maker of blood glucose control (161). In this study, there were no differences in relative kidney weight among the ZDF groups, even though there was a decrease in insulin resistance in the whole wheat or wheat fraction-fed groups. It is interesting that both whole wheat and refined wheat were able to improve insulin resistance to the same degree compared the control diet. This result brings into question the recommendation to consume whole grains over refined grains to reduce the risk of diabetes. Several clinical trials that examined the effect of whole grains versus refined grains also did not show improved glucose control (100-102). The inconsistent findings of improved insulin resistance and decreased BMI with whole grain consumption in observational studies and the lack of such an effect in clinical trials may be due to factors that were not controlled for such as healthier lifestyles in general. Participants who ate more whole grains in observational studies may have a better diet and may be more physically active (174). We suggest that the greater insulin sensitivity

of the whole wheat and the refined wheat groups, compared to the obese control, is due to the type of starch present. The obese control diet contained only purified corn starch whereas the whole wheat and refined wheat contained mostly wheat starch. Unpurified wheat starch contains proteins and more complex starch granules compared to purified corn starch (175). This, along with small amounts of soluble dietary fiber in the form of arabinoxylans, could slow glucose absorption, which is known to reduce the progression of insulin resistance (176, 177). The differences between the wheat diets and the control diet, which used corn starch as the main carbohydrate source, maybe be due to differences in the ratio of amylose to amylopectin, as amylose has a lower glycemic index than amylopectin (117). The percent of amylose and amylopectin in wheat starch is 25% and 75% respectively (178) whereas in corn starch it is 5% and 95% (124).

Although wheat bran was able to decrease insulin resistance, as shown by the decreased AUC during the ITT, there was not a corresponding change in the GTT or fasting blood glucose. This is consistent with a well-controlled study by Jenkins et al. (104), who found that consumption of wheat bran for 3 months did not alter measures of blood glucose control.

One characteristic of whole grains that has been suggested to improve insulin sensitivity is their low glycemic index. This is believed to improve glucose control by limiting large spikes in blood glucose and insulin (179). Interestingly, during the MTT in week 1, there was no difference in the AUC of the postprandial plasma glucose or insulin curves of the wheat diets compared to the obese control. This might suggest that the reduced insulin resistance observed in the wheat diets is not to be due to a lower glycemic index compared to the control diet. However, it may be that in this ZDF model the differences

in postprandial insulin and glucose caused by the wheat diets are too small to be observed from a meal tolerance test, but still result in improvements in glucose control by the end of 3 weeks of feeding.

It is unclear why wheat germ and high wheat germ improve insulin sensitivity and glucose control. The effect of wheat germ on insulin resistance is not widely studied, but in a clinical trial by Cara et al. (105), subjects who consumed 30 g of wheat germ for 4 weeks had no change in fasting blood glucose. There have been studies that suggest that particle size of the diet could be responsible for the differences in glucose control. It has been shown that larger particle sizes decrease postprandial glycaemia compared to smaller particle sizes (120, 180). However, a reduced glycemic response is not observed in all studies (121). In various animal studies it has been shown that food structure has an influence in gastric emptying, which reduces the rate of glucose entry into the bloodstream (180), therefore allowing circulating fatty acids to be used for energy (181). Increased circulating fatty acids are involved in the progression of peripheral and hepatic insulin resistance (182). Simply by not chewing food finely, whether the food is rice, potatoes or apples, will slow down glucose absorption, suggesting that the structure of food is more important than its composition (183). Larger particle sizes, such as wheat germ, have been shown to affect gastric emptying (184), however in this study a lower glycemic response was not observed and neither was it in other studies (185). Therefore, other explanations for the improved insulin resistance and glucose control are needed. There are data suggesting that wheat germ supplementation may reduce total lipid absorption (186) which could indirectly improve insulin resistance through decreased body fat. However, as we saw no difference in fat mass %, it is unclear how this may

have affected insulin resistance. There was no additive effect of the wheat fractions on glucose control because there was no difference between the wheat fraction groups and the whole wheat group.

Adipokines released by adipose tissue are associated with insulin resistance and fat mass. Circulating adiponectin, an adipokine that is generally inversely proportional to body fat, is generally reduced during insulin resistance (32, 187). Obese ZDF rats normally have lower concentrations of adiponectin compared to their lean counterparts (188), but in this study the obese control had significantly greater adiponectin concentrations compared to the lean rats. It is unclear why the obese control group would have greater adiponectin concentration compared to the lean and whole wheat groups, even though they have greater insulin resistance.

Resistin is released by adipose tissue (189) and has shown to be increased in animals with high body fat compared to lean animals (35). High plasma resistin levels have been associated with insulin resistance in humans (190), however other studies show no association (191). There was no significant difference in resistin levels among the ZDF groups, although there was a trend toward a decrease in the wheat groups compared to the ZDF control.

Oxidative stress can be classified as an imbalance between the formation of reactive oxygen species and the ability for the body to eliminate these reactive species that can cause damage to cellular macromolecules. These reactive species can trigger inflammatory pathways such as $\text{NF}\kappa\beta$ and JNK, which can activate kinases that interrupt insulin signaling, causing insulin resistance and T2D (192). A buildup of free radicals can

attack lipids, particularly unsaturated fatty acids, and cause peroxidation products to accumulate. Urinary 24 h TBARS were measured as a marker of whole body lipid peroxidation. Increased urinary TBARS is typically associated with greater insulin resistance, yet in this study, all wheat diets, except for wheat germ, had greater TBARS excretion compared to the obese control. It is unclear why the groups with lower insulin resistance had greater oxidative stress. Due to urine being collected in non-fasting rats, it is possible that other compounds in the wheat may have influenced the results of the TBARS assay.

NAFLD typically accompanies obesity and T2D and is characterized by an accumulation of triglyceride lipid droplets in hepatocytes. Increased fatty acid flux from enlarged adipose tissue as well as increased de-novo lipogenesis and/or increased fatty acid oxidation during hepatic insulin resistance, contributes to the accumulation of lipids (71). There was no difference in liver weight between the obese control and wheat groups, however, all whole wheat and wheat fraction diets had lower total liver lipids compared to the obese control, indicating a reduction in fatty liver. This is supported by previous studies, where the addition of whole wheat, refined wheat or wheat bran to the diet of rats reduced liver triglycerides (88). As mentioned above, a lower glucose and insulin response, either through small amounts of soluble fiber, the structure of starch granules, or particle size, could be the explanation. There is selective insulin resistance in the liver, where insulin fails to inhibit gluconeogenesis while at the same time is able to upregulate lipogenesis (74). This is in agreement with our observation of reduced glucose control, probably in part through increased gluconeogenesis, with greater fatty liver in the obese control group compared to the wheat diets. The decrease in total liver cholesterol

concentration in wheat bran, germ, and high germ groups may be due to greater fecal bile acid excretion, which was also greater in these groups. Bile acids are synthesized from cholesterol in the liver and therefore an increase in the excretion of bile acids results in the decrease of cholesterol in the liver (193) (194). This was not the case in the whole and refined wheat groups where there was no difference in liver cholesterol yet there was greater bile acid excretion. Previous studies show that cholesterol is decreased with soluble fiber consumption (195) and wheat fiber, which is mostly insoluble, has no effect (196). Another possible explanation for the decrease in liver cholesterol in the wheat germ groups could be the relatively high concentration of plant sterols. Due to the higher affinity of plant sterols for incorporation into the micelles, compared to cholesterol, there is reduced absorption of cholesterol from the intestinal lumen and therefore greater excretion in the feces (144). It appears that neither fiber content, which was mostly insoluble and balanced among all groups, nor bile acid excretion can entirely explain the decrease in total and free liver cholesterol.

Whole wheat contains high amounts of ferulic acid, a phenolic compound that may have a beneficial role in reducing body weight (140) and slowing the progression of T2D (135). Ferulic acid is able to form a stable radical, allowing it to scavenge reactive oxygen species (ROS) produced as byproducts in the electron transport chain. ROS have been implicated in insulin resistance by interfering with normal insulin signaling pathways and triggering inflammation in the muscle and liver (139). Ferulic acid is mostly bound to the cell wall of wheat and therefore is only slightly bioavailable due to limited release by gut microflora (138). The whole wheat and wheat bran groups, which had the greatest amount of ferulic acid absorbed, based on urinary ferulic acid excretion,

showed improvements in glucose control and insulin resistance. On the other hand, this may not be due to ferulic acid absorption because the wheat germ, which showed little urinary ferulic acid excretion, also showed decreased insulin resistance. In fact, wheat bran, which is the milling fraction containing the most ferulic acid, did not cause a difference in GTT AUC or fasting blood glucose. These results do not support the suggestion that insulin resistance is altered by ferulic acid in wheat as all the wheat diets showed an improvement in glucose control.

In conclusion, the consumption of whole wheat and its milling fractions for 5 weeks improved glucose control and decrease insulin resistance and fatty liver in ZDF rats. Whole wheat, however, was no more effective at improving these parameters than its individual milling fractions. These effects do not appear to be due to changes in plasma adiponectin concentration or absorption of ferulic acid. It is possible that small amounts of soluble fiber in the whole wheat and refined wheat fraction may be responsible for the positive health benefits, but it is unclear why the bran and germ fractions were also equally effective. Thus, in this short term study, none of the wheat milling fractions stand out as responsible for the effects seen with consumption of whole wheat. The results of this study suggest that individual milling fractions of wheat are just as effective in improving insulin resistance and fatty liver as whole wheat.

Advantages and limitations of the study and future directions

This study has many advantages, such as a well-controlled study design, use of a food source in a form that is normally consumed in the population rather than a food extract, a balanced macronutrient composition, and an examination of each milling fraction in

addition to whole wheat. To our knowledge, this is the first study to do this. That said, there are also some limitations. One unavoidable limitation was the length of the study. It would have been helpful to prolong the feeding trial to see long-term effects of the diets, but due to the characteristics of the animal model, the rats would have been in an advanced state of diabetes and it would have been impossible to see changes brought on by the diet. Even in this short term study the animals may have been too diabetic and obese, making it more difficult to detect large changes between the groups. Finally, as with all animal studies, to what degree the results can be extrapolated to humans is uncertain.

Following the results acquired from this study, future research can be directed in various ways to further explore possible mechanisms of whole wheat and its milling fractions. First, the effect of particle size on postprandial glucose curve could be examined by milling whole wheat into different particle sizes and observing whether there is a difference in the parameters we studied. Another approach could be varying the ratios of amylose to amylopectin using different wheat varieties to determine its effects on insulin resistance and glucose control. Using a different animal model, such as the high fat-fed rat, and feeding for a longer period of time could also be helpful in finding small differences that may not have been detectable with the ZDF model.

Table 2-1. Diet composition

Nutrient (g/kg)	Control	Whole wheat flour	Refined wheat flour	Wheat bran	Wheat germ	High Wheat Germ
Flour	0	650	542.75	0	0	0.00
Milling fraction	0	0	0	94.25	16.25	153.75
Sucrose	40	40	40	40	40	40.00
Corn Starch	506.95	5.4	59.35	463.45	498.08	424.50
Soybean Oil	120	105.50	100.18	114.29	118.17	105.00
Casein	200	136.28	149.6	188.31	195.92	164.00
Cellulose	81.3	11.06	56.36	47.93	79.81	61.00
Mineral Mix	35	35	35	35	35	35
Vitamin Mix	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3
Cholesterol	1.25	1.25	1.25	1.25	1.25	1.25
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
BHT	0.014	0.014	0.014	0.014	0.014	0.014
Total Weight	1000.014	1000.014	1000.014	1000.014	1000.014	1000.014
% CHO	54.70	53.86	52.65	56.35	54.57	53.66
% Protein	20.00	18.90	18.78	20.00	20.00	20.26
% Fiber	8.13	6.83	7.05	8.13	8.13	7.51
% Fat	12.00	12.00	10.84	12.00	12.00	12.23

Table 2-2. Measures of body weight, food intake, tissue weights, cecum pH, glucose control

Parameters	Negative Control	Positive Control	Whole Wheat	Refined Wheat	Wheat Bran	Wheat Germ	High Wheat Germ
Weight Gain (g)	104.6 ± 5.8 ^a	158.4 ± 3.1 ^{bc}	148.7 ± 6.3 ^b	153.1 ± 3.8 ^{bc}	158.4 ± 3.7 ^{bc}	161 ± 3.6 ^c	160.8 ± 2.7 ^c
Average food intake (g)	16.7 ± 0.6 ^a	22.7 ± 1.04 ^b	22.7 ± 0.41 ^b	23.1 ± 0.53 ^b	23.1 ± 0.7 ^b	24.5 ± 0.82 ^b	23.4 ± 0.33 ^b
Cecal pH	6.8 ± 0.07 ^b	6.8 ± 0.07 ^b	6.2 ± 0.11 ^a	6.9 ± 0.04 ^b	6.8 ± 0.08 ^b	6.8 ± 0.09 ^b	6.7 ± 0.05 ^b
Cecum weight (g)	0.389 ± 0.007 ^{bc}	0.337 ± 0.010 ^a	0.525 ± 0.021 ^d	0.439 ± 0.014 ^c	0.374 ± 0.020 ^{ab}	0.390 ± 0.012 ^b	0.410 ± 0.018 ^{bc}
Epididymal fat pad weight (g)	0.405 ± 0.011 ^a	1.149 ± 0.055 ^b	1.144 ± 0.032 ^{bc}	1.091 ± 0.015 ^b	1.154 ± 0.031 ^{bc}	1.129 ± 0.021 ^{bc}	1.149 ± 0.021 ^c
Retroperitoneal + perirenal fat pad weight (g)	0.492 ± 0.029 ^a	1.467 ± 0.027 ^b	1.464 ± 0.052 ^b	1.471 ± 0.0528 ^b	1.513 ± 0.049 ^b	1.469 ± 0.040 ^b	1.583 ± 0.023 ^b
Inguinal fat pad weight (g)	0.672 ± 0.031 ^a	3.443 ± 0.201 ^b	3.146 ± 0.184 ^b	3.293 ± 0.162 ^b	3.236 ± 0.194 ^b	3.442 ± 0.120 ^b	3.560 ± 0.155 ^b
Body fat % ²	18.95 ± 0.56 ^a	55.15 ± 1.31 ^b	51.75 ± 0.72 ^{b*}	53.64 ± 1.27 ^b	55.38 ± 1.95 ^b	55.7 ± 1.66 ^b	54.89 ± 2.09 ^b
Lean mass % ²	81.027 ± 0.55 ^b	44.88 ± 1.29 ^a	48.20 ± 0.71 ^a	46.378 ± 1.26 ^a	44.60 ± 1.97 ^a	44.33 ± 1.69 ^a	45.13 ± 2.09 ^a
Fasting blood glucose week	98.1 ± 4.2 ^a	149.6 ± 6.4 ^c	125.6 ± 5.3 ^b	121.3 ± 4.8 ^b	136.1 ± 4.7 ^{bc}	134.1 ± 3.2 ^b	136.2 ± 6.4 ^{bc}

3 (mg/dL)							
AUC GTT (x10 ³)	18.76 ± 0.33 ^a	32.34 ± 1.238 ^c	27.54 ± 1.45 ^b	28.19 ± 0.68 ^b	30.16 ± 1.28 ^{bc}	28.62 ± 0.9 ^b	29.13 ± 1.16 ^b
AUC ITT (x10 ³)	8.26 ± 0.92 ^a	22.47 ± 0.8 ^c	19.1 ± 0.79 ^b	19.61 ± 0.64 ^b	19.91 ± 0.79 ^b	20.04 ± 0.55 ^b	19.24 ± 0.67 ^b
AUC MTT Glucose response (x10 ³)	26.94 ± 0.34 ^a	32.99 ± 0.95 ^{bc}	30.48 ± 1.68 ^{bc}	31.98 ± 0.91 ^{bc}	33.74 ± 1.34 ^c	32.57 ± 0.74 ^{bc}	29.84 ± 1.21 ^{ab}
AUC MTT Insulin response	105.51 ± 15.67 ^a	804.72 ± 114.35 ^{bc}	758.09 ± 125.79 ^{bc}	862.73 ± 134.07 ^c	817.65 ± 132.30 ^{bc}	863.48 ± 101.36 ^c	530.98 ± 125.41 ^b
Kidney weight, as a percentage of body weight (g/100g BW)	0.703 ± 0.013 ^b	0.622 ± 0.059 ^a	0.671 ± 0.017 ^a	0.630 ± 0.016 ^a	0.618 ± 0.019 ^a	0.606 ± 0.015 ^a	0.622 ± 0.217 ^a
TBARS (µg/24h)	3.586 ± 0.384 ^a	6.002 ± 0.361 ^b	12.183 ± 1.158 ^e	8.367 ± 0.771 ^c	8.827 ± 0.585 ^{cd}	8.111 ± 0.622 ^{bc}	10.810 ± 0.899 ^{de}
Adiponectin (µg/ml)	28.559 ± 1.61 ^a	35.668 ± 2.448 ^c	29.228 ± 2.061 ^{ab}	37.394 ± 2.506 ^c	32.270 ± 2.292 ^{abc}	36.789 ± 2.409 ^c	34.518 ± 3.047 ^{bc}
Resistin (ng/ml)	16.6 ± 1.29 ^a	20.4 ± 0.84 ^b	18.8 ± 1.1 ^{ab}	18.6 ± 1.06 ^{ab}	18.3 ± 1.33 ^{ab}	18.31 ± 0.94 ^{ab}	18.7 ± 1.04 ^{ab}

¹Values represent mean ± SEM, n=10-12, except where noted. Values within a row that do not share a common superscript are significantly different, p<0.05.

²Measured at 4 weeks.

*Significantly different from obese control by Student's t-test.

Table 2-3. Liver weights, liver lipids, liver cholesterol bile acid excretion

Parameter	Negative Control	Positive Control	Whole Wheat	Refined Wheat	Wheat Bran	Wheat Germ	High Wheat Germ
Liver weight (g)	7.421 ± 0.276 ^a	15.058 ± 0.625 ^b	15.766 ± 0.062 ^b	14.488 ± 0.437 ^b	14.527 ± 0.740 ^b	14.116 ± 0.619 ^b	14.606 ± 0.913 ^b
Total liver lipids (mg/liver)	527.0 ± 43.8 ^a	2583.5 ± 294.5 ^c	1844.2 ± 120.4 ^b	1694.9 ± 152.4 ^b	1491.1 ± 157.9 ^b	1605.7 ± 180.3 ^b	1479.9 ± 170.9 ^b
Total liver cholesterol (mg/g)	7.003 ± 0.372 ^a	13.361 ± 0.922 ^d	10.86 ± 0.733 ^{bcd}	11.05 ± 0.969 ^{cd}	8.513 ± 0.679 ^{ab}	9.454 ± 0.627 ^{abc}	6.939 ± 0.677 ^a
Free liver Cholesterol (mg/g)	2.029 ± 0.02 ^b	1.875 ± 0.05 ^{ab}	1.701 ± 0.04 ^a	1.862 ± 0.08 ^a	1.74 ± 0.041 ^a	1.87 ± 0.05 ^{ab}	1.77 ± 0.05 ^a
Bile acid excretion (mg/day)	3.262 ± 0.119 ^{ab}	2.915 ± 0.178 ^a	5.586 ± 0.525 ^c	3.831 ± 0.156 ^b	5.022 ± 0.406 ^c	3.970 ± 0.179 ^b	3.994 ± 0.148 ^b

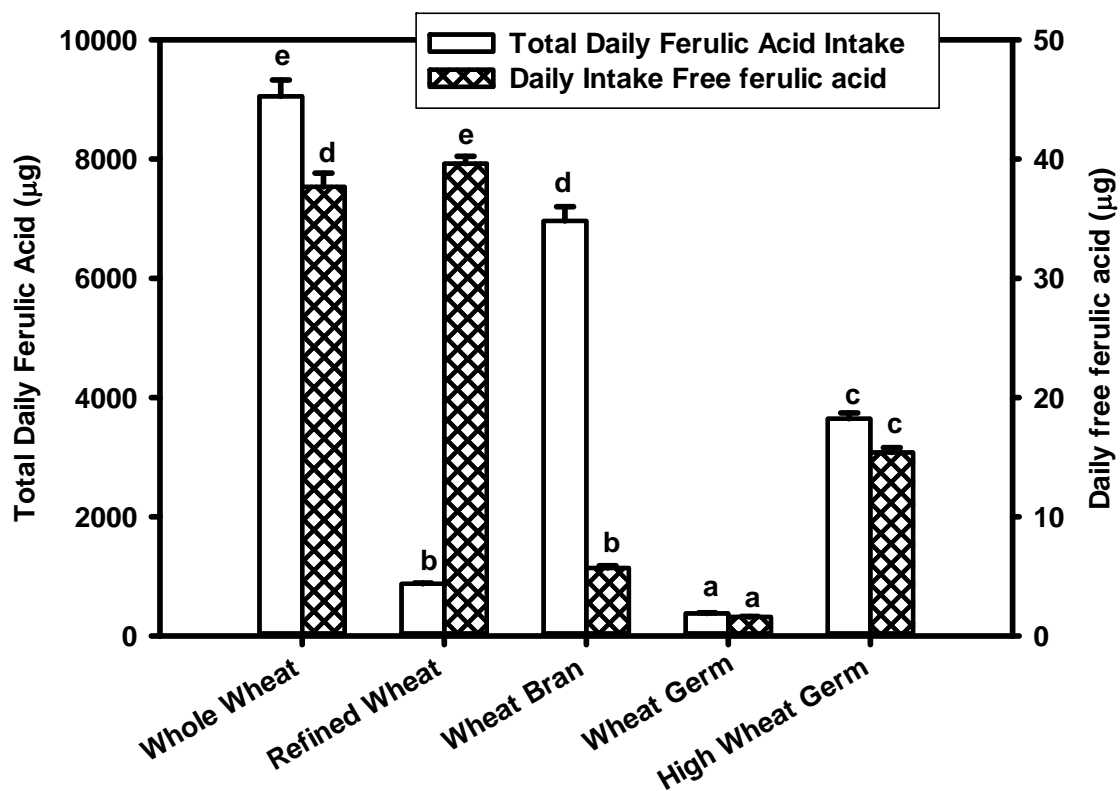


Figure 2-1. Daily total and free ferulic acid intake

Values represent mean \pm SEM, n=10-12. Values that do not share a common superscript are significantly different, $p < 0.05$. Measured at 4 weeks.

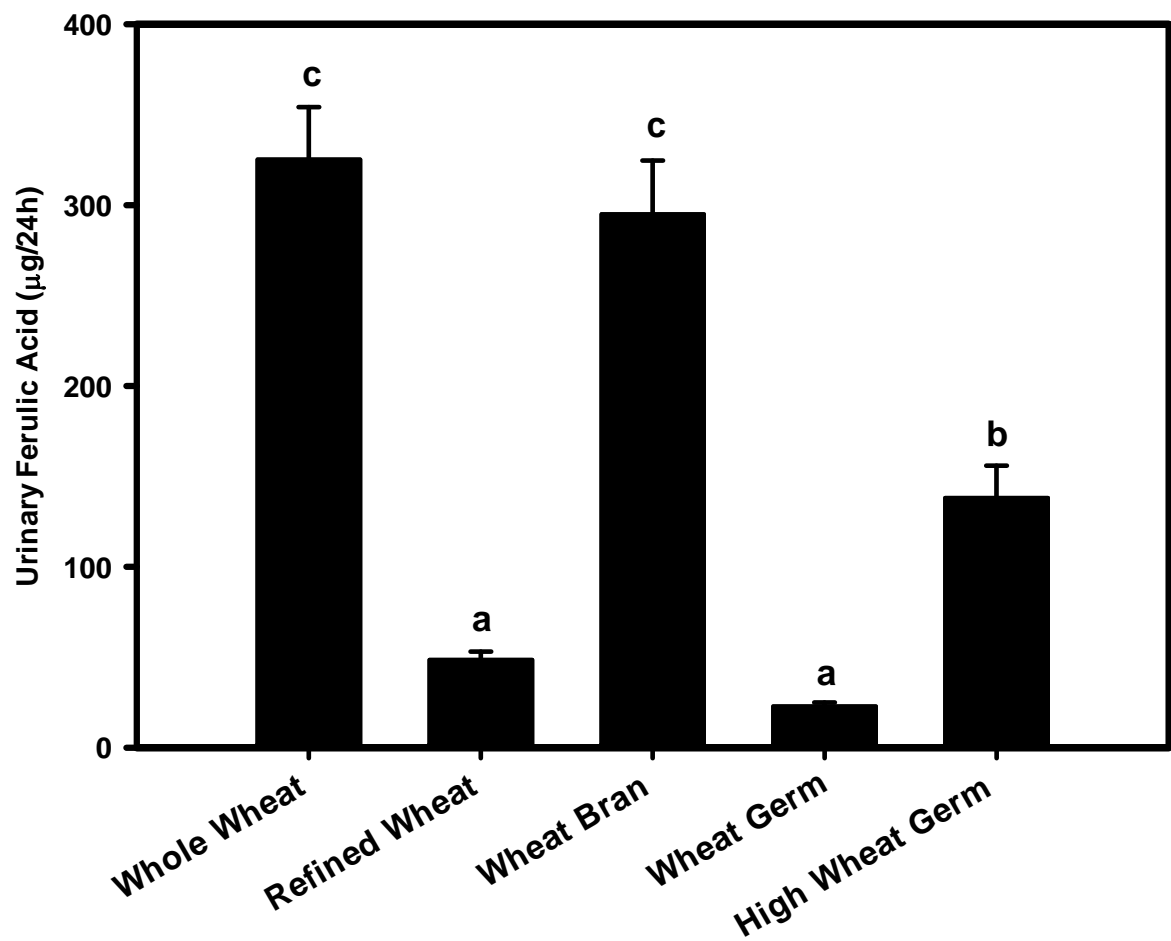


Figure 2-2. Urinary excretion of ferulic acid over 24 h

Values represent mean \pm SEM, n=10-12. Values that do not share a common superscript are significantly different, $p < 0.05$. Measured at 4 weeks.

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Appendices

Appendix 1: Liver Lipid Extraction

- 1) Weigh out ~1.00 gram of liver tissue and transfer to homogenizing tube.
- 2) Add 10 mL Chloroform:MeOH (2:1).
- 3) Homogenize for ~15-20 seconds.
- 4) Filter homogenate through a #1 Whatman filter paper into a glass screw top tube.
- 5) Rinse homogenizer with 15 ml Chloroform:MeOH (2:1) and pick out any tissue trapped on probe.
- 6) Pour rinse through filter paper into tube.
- 7) Wash filter paper with an additional 5 ml Chloroform:MeOH (2:1).
- 8) Add 6 ml 0.9% NaCl to filtrate. This represents a volume of 0.2 times the volume of Chloroform:MeOH used.
- 9) Vortex to mix aqueous and nonaqueous phases.
- 10) Centrifuge for 1 minutes at 750 RPM to separate phases. Be careful about placement of tubes in the centrifuge – when rotor spins, long tubes may break as they tilt.
Alternatively, let the tubes stand until phases separate.
- 11) Remove aqueous upper phase with a transfer pipet and discard.
- 12) Gently layer 3 ml of MeOH:H₂O (1:1). Rinse sides of tubes while adding the MeOH:H₂O.
- 13) Remove this upper phase with a transfer pipet and discard.
- 14) Transfer the extracted lipid in Chloroform:MeOH (2:1) and transfer rinse to lipid vial.
- 15) Evaporate Chloroform under N₂ gas. (Gravimetric Step)

16) Reconstitute the fat with a set volume for all samples (10 ml) in Chloroform:MeOH (2:1).

Appendix 2: Enzymatic Cholesterol Assay

Reagents

1) 50 **mM Sodium Phosphate, Dibasic** (MW = 141.96). Make 1 L, pH 6.9

(7.1 g/L).

2) Stock **Phenol Reagent**: 500 ml

Dissolve 3.8 g Phenol crystals in 400 ml Phosphate Buffer. Adjust volume to 500 ml with Phosphate Buffer. Store at 4degrees? C (up to 1 month).

3) Stock **Mixed Reagent**: 500 ml

Dissolve in 400 ml Phosphate Buffer:

0.203 g 4-aminoantipyrine

1.292 g Sodium cholate

7.46 g Potassium chloride

1.0 ml Triton X

Adjust volume to 500 ml with Phosphate Buffer. Store at 4° C (up to 1 month).

4) **Working Reagent**: (shelf life: 3-4 days)

	(UI)/30mL	(UI)/100 mL	(UI)/200 mL
<u>Reagent</u>	<u>(mL)</u>	<u>(mL)</u>	<u>(mL)</u>
Stock Mixed Reagent	15	50	100
Stock Phenol	15	50	100
Cholesterol Oxidase (UI)	7.5	25	50
Cholesterol Esterase (UI)	7.5	25	50

Peroxidase (UI)	375	1250	2500
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Store in ice bath until ready to use.

******NOTE**** For free cholesterol assay, omit esterase**

A) Standard Curve:

Use 1 mg/ml concentration of cholesterol in Chloroform:MeOH (2:1). Use 0, 5, 10, 15, 20, and 25 μ l concentrations (depends on cholesterol in sample).

Add 50 μ l Triton X-100/Acetone to each tube, Vortex.

Dry under N₂ gas. (Triton Sol'n = 1.5 g Triton in 10 mL acetone)

B) Samples: Total Cholesterol: 10 μ l assayed

Free Cholesterol: 50 μ l assayed

Add 50 μ l Triton X-100/Acetone.

Dry solvent under N₂ gas

C) Assay: Add 100 μ l ddH₂O to each tube, Vortex.

Add 1 ml Working Reagent/sample, Vortex.

Incubate at 37° C for 10 min. Cool to room temperature.

Read absorbance at 500 nm.

Appendix 3: Assay of Total Bile Acids

References:

1. MacDonald, Ian (1976) Clin. Biochem. 9, (3) 153-154, Enzymatic Analysis of Bile Acids.
2. Locket, P.L., Gallaher, D.D. (1989), Lipids 24 221-223
3. Bile Acid Assay III (Sheltawy, 1975, Determination of faecal bile acids by an enzymic method)

Materials:

Equipment

Spectrophotometer

Waterbath: Temp. approx. 37° C

Reagents

0.01M Cholic Acid in 100% MeOH for std. Curve

3 α -Hydroxysteroid dehydrogenase; Sigma No. H-1506 from

Pseudomonas testosteroni; Need ~1.5 units enzyme/mL phosphate buffer, but could go **down to 1.25 units/mL**

- 0.01M Phosphate buffer; use sodium phosphate monobasic, pH 7.2
- 0.05M CAPS buffer, adjust pH to 10.8 w/ NaOH
- NAD (β -nicotinamide adenine dinucleotide), use 1.25mg NAD/ml CAPS buffer. Use NAD soln the day it is made
- Methanol, reagent grade

PROCEDURE

Generation of Standard Curve

A standard curve must be generated each day that sample bile acids are run. This is because the quantity of bile acids in each sample is calculated from the equation of the regression line generated by the standard curve. This equation will vary with minor changes in the concentration of NAD substrate and enzyme. Because new NAD soln must be made with each assay a new std. curve must also be run. No more than 30 samples should be run per one standard curve. **STANDARD CURVE SHOULD BE DONE IN TRIPLICATE.**

1. Label small test tubes with vol. of cholic acid std. to be added. Use std. volumes of 0, 10, 20, 30, 40, and 50ul.

2. To each tube add:

100% MeOH to give a total MeOH volume (Std. vol. + MeOH) of 100 μ l. (i.e. to tubes labelled 0 add 100 μ l MeOH)

Cholic acid standard in MeOH, volumes listed in step 1

1.25 ml NAD in CAPS buffer

150 μ l Enzyme solution

3. Cap tubes with corks or cover the rack with parafilm tightly and vortex.

4. Incubate the tubes in a water bath at approx. 37 $^{\circ}$ C for 20 min.

5. Remove tubes from water bath and allow them to adjust to room temperature for about 5 min.

Sample Preparation

After you have determined that the bile acid assay works, (a standard curve has been run independently and proves linear) samples should be prepared for bile acid analysis. Run a standard curve each day that you run samples.

1. Resolubilize partially purified bile acids in 0.5ml 100% MeOH.

2. **Add to assay tubes**

50 µl 100% MeOH

50 µl sample

1.25ml NAD in CAPS

150 µl Enzyme soln

3. Cap samples, vortex then incubate samples with standards.

*Note: if absorbance is too high or low adjust sample volume assayed to bring absorbance to around 1.

Spectrophotometric analysis

Set up a program on the computer attached to the spec for this assay. Note the wavelength of **absorbance is 340 nm. Add a couple blank analysis samples at end for repetition if needed.**

Zero the instrument using double distilled water. Use the 0 level bile acid standard as your blank.

Run standards followed by samples. Rerun the standard blanks at end of run.

Calculation of Bile Acid Quantity

1. Convert μl Std. to nmoles B.A.

2. Run a regression of nmoles B.A. against Corrected Absorbance.

Corrected Absorbance = $(\text{Abs. 1} - \text{Abs. 2})/2 - \text{Ave Abs. Blank}$

3. Y-intercept should be close to zero, $r^2 = 0.99$

4. From the corrected absorbances of samples (ave. of 2 sample reps - blank) calculate nmoles B.A. using regression equation.

July 28, 1992, June 2004

C. Gallaher/DDG

Bile Acid Extraction and Purification

Reference: Locket, PL & Gallaher, DD (1989) Lipids 24, 221-223.

MATERIALS

Methanol

Ethanol, absolute

Chloroform

Petroleum Ether

Solid phase extraction columns, reverse phase C18

Screw-capped tubes (check to make sure they seal tight)

Bath sonicator

Heating block

Nitrogen gas

SOLUTIONS

Ethanol, 80 %

Add 20 parts of distilled water to 80 part of absolute ethanol. Store at room temperature.

Chloroform/methanol

Mix equal volumes of chloroform and methanol. Store at room temperature.

PROCEDURE

Extraction

1. Weigh approximately 100 mg of tissue or feces into a screw cap tube (20 X 125 mm or similar size). Record the exact weight.
 2. Add 2 ml of absolute ethanol to each tube.
 3. Sonicate the capped tubes in a bath sonicator for 30 min.
 3. Place the tubes in a heating block and reflux at 100°C for 15 min.
 4. Centrifuge the tubes at approx. 1,500 X g for 10 min. Transfer the supernatants to screw capped tubes.
 5. Suspend the pellets in 2 ml of 80% ethanol, reflux for 15 min in the heating block, and centrifuge as in step 4. Again transfer the supernatants to the screw capped tubes.
 6. Suspend the pellet again in 2 ml of chloroform/methanol, reflux for 15 min in the heating block, and centrifuge as in step 4. Transfer the supernatants to the screw capped tubes.
- Suspend the pellet in 2 ml of chloroform/methanol, centrifuge as in step 4, and transfer the supernatants to the screw capped tubes.
8. Evaporate the pooled supernatants to dryness with nitrogen gas under low heat (<60°C). Store dried samples capped in the freezer until ready for partial purification.

Partial Purification

1. Remove the rack, if present, from inside the solid phase extraction (SPE) column processor. Insert the SPE columns into the column processor and turn on the water aspirator. Adjust the vacuum to about 5-10 mm Hg with the screw located on the vacuum arm closest to the processor (NOT the wing nut).

2. Solvate (activate) the columns by adding 4 ml of MeOH to each column. Follow with 4 ml of distilled water (DW). Do not allow the columns to dry out if possible between solutions. Stop the flow through the columns by releasing the vacuum by opening the wing nut.
3. To tubes containing the dried tissue or fecal extract, add 0.6 ml MeOH and vortex to solubilize the bile acids. Next add 2.4 ml DW to each tube and vortex again.
4. Pour the solution from above through a solvated column and apply vacuum.
5. When the level of solution in the columns drops to the frit, add 3 ml of DW.
6. When the level of DW in the columns drops to the frit, add 3 ml of hexane.
7. When the level of hexane has dropped to the frit for all columns, release the vacuum with the wing nut and carefully remove the cover of the SPE column processor. Insert the rack with labeled tubes in the correct position to collect the column eluents.
8. Add 3 ml MeOH to each column and turn on the vacuum. The flow of MeOH through the column should be 2-3 ml/min. The MeOH will elute the bile acids off the columns, which will then be collected in the tubes within the processor.
9. After the flow through the columns stops, release the vacuum, remove the tubes, and evaporate the solution to dryness with nitrogen gas at low heat.

Appendix 4: Urinary Thiobarbituric Acid Reactive Substances

(TBARS) Procedure

Reference: Hye-Sung Lee, *Lipids* 27:124-128, 1992

Reagents:

- 5% (w/v) Tri-chloroacetic acid (TCA). 5g to 100 ml H₂O.
- 0.06 M Thiobarbituric acid (TBA). 0.8652g 2-thiobarbituric acid to 100 ml H₂O.

- ❖ Store stoppered reagents in cold room.
- ❖ Before use, warm TBA solution in water bath (as the bath heats up) to return all precipitate to solution, then cool to room temperature.

MDA Standard:

- 10⁻⁵ M Malondialdehyde (MDA)
- **Synthesis of MDA**
 1. Weigh 164.2 mg (1 mmol) malondialdehyde tetramethylacetal into a 100 ml glass stoppered Erlenmeyer flask.
 2. Add 100 ml 0.01 N HCl, stopper immediately and wrap the top with Parafilm.
 3. Heat in 50 °C water bath for 1 hr.
 4. Cool to room temperature in a dark place.
 5. Dilute with water to 10⁻⁵ M.
 - 1 ml 10⁻² M (as prepared) to 100 ml water = 10⁻⁴ M
 - 10 ml 10⁻⁴ M to 100 ml water = 10⁻⁵ M.

- ❖ MDA is very sensitive to light, store in the cold room in a flask wrapped in a foil.
- ❖ Approximate shelf-life is one month.

Appendix 5: Ferulic Acid

Urinary ferulic acid was measured by HPLC after deconjugation using a modification of the method of Bunzel et al. Briefly, ferulic acid was deconjugated by mixing 5% of 24 h-collected rat urine with the same volume of 1 M sodium acetate buffer (pH 4.9), 300uL of sulfatase solution, and 100 μ L of 0.1 mg/mL alpha coumaric acid solution (in 50% aqueous methanol). The mixture was incubated 37 °C for 3 h. The reaction was stopped with concentrated hydrochloric acid 0.3 mL and ferulic acid extracted with a total of 4mL ethyl acetate (2mL, 1mL, 1mL). The pooled ethyl acetate phases were extracted with 5ml of 5% sodium hydrogen carbonate (2mL, 2x 21.5mL) and phases separated by centrifugation. The combined sodium hydrogen carbonate phases were acidified with 0.5mL of concentrated hydrochloric acid until the pH was <2, and then extracted with 3 x 2.5mL ethyl acetate. The ethyl acetate phases were collected and dried under nitrogen. The dried extract was reconstituted in 200uL 50% methanol and 1:5 diluted for HPLC analysis.

Quantification of ferulic acid was achieved using a Shimadzu HPLC system with auto sampler and SPD-M20A PDA detector. HPLC conditions are as follows: A Luna Phenyl-Hexyl column (250 x10mm; 4.6um particle size) was used for separation at a flow rate of 1mL/min and temperature of 45 °C. Mobile phases were 0.1 mM trifluoroacetic acid (TFA) (mobile phase A), 90% methanol/10% TFA (mobile phase B) and 90% acetonitrile/10% TFA (mobile phase C). The following gradient program was used (156): 87% A and 13% C for 10 min; change over 10 min to 77% A, 3% B, and 20% C; change

over 5 min to 70% A, 5% B, and 25% C; and finally change over 5 min to 25% A, 25% B, and 50% C. Detection wavelengths were 280 and 325 nm.

Appendix 4: SAS CODE

```
data all_groups    (label = 'All diet groups')
zdf_only (label= 'ZDF Animals Only')
;
Title2 'Effect of Wheat and Wheat Fractions in ZDF Rats';
Input Rat_No Block_No Diet_Group Initial_BW Week_1_BW Week_2_BW
Week_3_BW MidWeek_4_BW Week_5_BW Total_Weight_Gain Week1_FI Week2_FI
Week3_FI Average_FI MTT_Time0 Mtt_Time15 MTT_Time30 MTT_Time60
MTT_Time90 MTT_Time120
MTT_Time180 BodyWeight_GTT Amount_Gavaged_GTT GTT_Time0 GTT_Time15
GTT_Time30 GTT_Time60 GTT_Time90 GTT_Time120 GTT_Time180 ITT_Time0
ITT_Time15 ITT_Time30 ITT_Time60 ITT_Time90 ITT_Time120
ITT_Time180 Urine_Vol Fecal_DryWeight TissuePCFat_DXA
Body_Tissue_Weight_DXA Body_Fat_DXA Lean_BW_DXA TissuePCLean_DXA
BMD_DXA BMC_DXA
KillDay_BW KillDay_Liver_Weight KillDay_Kidney_Weight Kidney_PC
      KillDay_Epi_fatpad_Weight EpifatPC Retro_perinal_fatpad
Retro_perinal_fatpadPC Killday_Inguinal_fatpad_weight
InguinalfatPC Cecum_weight CecumPH Total_LiverLipids_assayed
Lipids_lg_tissue Liverlipids_whole_liver MTT_AUC GTT_AUC ITT_AUC
Liver_total_chol_conc_true Liver_total_chol_true
Liver_free_chol_conc_true Liver_free_chol_true
fecal_wt_assayed adiponectin_conc pCA FA diOHpropA OH3MepropA
resistin_sample_ngml fecal_bile_acid TBARS_ug24hr T0_MT_insulin
T30_MT_insulin T60_MMT_insulin T120_MMT_insulin T180_MMT_insulin
AUC_MMT_Insulin

;

Label
Rat_No = 'Rat Number'
Block_No= 'Block Number'
Diet_Group= 'Diet Group'
Initial_BW = 'Initial Body Weight (g)'
Week_1_BW= 'Week 1 Body Weight (g)'
Week_2_BW= 'Week 2 Body Weight (g)'
Week_3_BW = 'Week 3 Body Weight (g)'
MidWeek_4_BW = ' Mid Week 4 Body Weight (g)'
Week_5_BW = 'Week 5 Body weight (g)'
Total_Weight_Gain = 'Total Weight Gain (g)'
Week1_FI= ' Food Intake Week 1 (g)'
Week2_FI = 'Food Intake Week 2 (g)'
Week3_FI = 'Food Intake Week 3 (g)'
Average_FI= 'Average Food Intake (g)'
MTT_Time0 = 'Meal Tolerance Test,Blood Glucose at Time 0'
Mtt_Time15 = ' Meal Tolerance Test,Blood Glucose at 15 minutes (mg/dL)'
MTT_Time30 = ' Meal Tolerance Test,Blood Glucose at 30 minutes (mg/dL)'
MTT_Time60 = ' Meal Tolerance Test,Blood Glucose at 60 minutes(mg/dL)'
MTT_Time90 = ' Meal Tolerance Test,Blood Glucose at 90 minutes(mg/dL)'
MTT_Time120= ' Meal Tolerance Test,Blood Glucose at 120 minutes(mg/dL)'
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MTT_Time180=' Meal Tolerance Test,Blood Glucose at 180 minutes(mg/dL)'
 BodyWeight_GTT= 'Body Weight Measurement for Glucose Tolerance Test (g)'
 Amount_Gavaged_GTT=' Amount of Glucose Gavaged During the Glucose Tolerance Test (ml)'
 GTT_Time0 = 'Glucose Tolerance Test, Blood Glucose Level at Time 0 (ml/dL)'
 GTT_Time15 ='Glucose Tolerance Test, Blood Glucose Level at Time 15 (ml/dL)'
 GTT_Time30='Glucose Tolerance Test, Blood Glucose Level at Time 30 (ml/dL)'
 GTT_Time60 ='Glucose Tolerance Test, Blood Glucose Level at Time 60 (ml/dL)'
 GTT_Time90 ='Glucose Tolerance Test, Blood Glucose Level at Time 90 (ml/dL)'
 GTT_Time120 ='Glucose Tolerance Test, Blood Glucose Level at Time 120 (ml/dL)'
 GTT_Time180 ='Glucose Tolerance Test, Blood Glucose Level at Time 180 (ml/dL)'
 ITT_Time0=' Insulin Tolerance Test,Blood Glucose at 0 Minute (ml/dL)'
 ITT_Time15=' Insulin Tolerance Test,Blood Glucose at 15 Minute (ml/dL)'
 ITT_Time30=' Insulin Tolerance Test,Blood Glucose at 30 Minute (ml/dL)'
 ITT_Time60 =' Insulin Tolerance Test,Blood Glucose at 60 Minute (ml/dL)'
 ITT_Time90 =' Insulin Tolerance Test,Blood Glucose at 90 Minute (ml/dL)'
 ITT_Time120=' Insulin Tolerance Test,Blood Glucose at 120 Minute (ml/dL)'
 ITT_Time180=' Insulin Tolerance Test,Blood Glucose at 180 Minute (ml/dL)'
 Urine_Vol = 'Urine Volume over 24h Period (ml)'
 Fecal_DryWeight = 'Dry Weight of 48h Fecal Collection (g)'
 TissuePCFat_DXA= '% of Tissue Fat Obtained From DEXA Scanning'
 Body_Tissue_Weight_DXA= 'Body Tissue Weight Obtained by DEXA (g)'
 Body_Fat_DXA ='Total Rat Body Fat Calculated by DEXA (g)'
 Lean_BW_DXA=' Lean Body Weight Calculated by DEXA (g)'
 TissuePCLean_DXA= '% Of lean Tissue of Rat calculated by DEXA (g)'
 BMD_DXA = 'Bone Mineral Density From DEXA, (g/cm2)'
 BMC_DXA= 'Bone Mineral Content (g)'
 KillDay_BW= 'Rat Body Weight At Kill Day (g)'
 KillDay_Liver_Weight= 'Rat Liver Weight at Kill day (g)'
 KillDay_Kidney_Weight= ' Rat Kidney Weight at Kill day (g)'
 Kidney_PC = 'Kidney weight as a% of body weight'
 KillDay_Epi_fatpad_Weight= 'Kill day Epididymal Fat Pad Weight(g)'
 EpifatPC= 'Epididymal Fat Pad weight as a% of body weight'
 Retro_perinal_fatpad = 'Kill Day Retroperitoneal and Perirenal Fat Pad Weight (g)'
 Retro_perinal_fatpadPC= ' Retroperitoneal and Perirenal weight as a% of body weight'
 Killday_Inguinal_fatpad_weight= ' Inguinal Fat Pad Weight Kill Day (g)'
 InguinalfatPC = ' Inguinal Fat Pad weight as a% of body weight'
 Cecum_weight = ' Cecum Weight At Kill day (g)'
 CecumPH = ' Cecum pH level at Kill day'
 Total_LiverLipids_assayed='Total Liver Lipids assayed'
 Lipids_lg_tissue= ' Total Liver lipids per Gram of Liver Tissue (ml/g L)'
 Liverlipids_whole_liver='Total liver lipids in whole liver (mg/liver)'

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MTT_AUC='Meal tolerance test AUC'
GTT_AUC='Glucose tolerance test AUC'
ITT_AUC='Insulin tolerance test AUC'
Liver_total_chol_conc_true='Liver total cholesterol conc true (mg/g)'
Liver_total_chol_true='Liver total cholesterol per liver (mg/liver)'
Liver_free_chol_conc_true='Liver free cholesterol conc true (mg/g)'
Liver_free_chol_true='Liver free cholesterol per liver (mg/liver)'
fecal_wt_assayed='Fecal wt assayed'
adiponectin_conc='Plasma fasting adiponectin (ug/mL)'
pCA='urinary total p-caffeic acid (ug/24h)'
FA='urinary total ferulic acid (ug/24h)'
diOHpropA='urinary total 3-(3,4-dihydroxy-phenyl)propA (ug/24h)'
OH3MepropA='urinary total 3-(4-hydroxy-3-methoxy-phenyl)propA (ug/24h)'
Tot_FA_ref_flour='Total ferulic acid in refined flour (ug/g flour)'
Tot_FA_WW_flour='Total ferulic acid in whole wheat flour (ug/g flour)'
Tot_FA_bran='Total ferulic acid in wheat bran (ug/g bran)'
Tot_FA_germ='Total ferulic acid in wheat germ (ug/g germ)'
Free_FA_ref_flour='Free ferulic acid in refined flour (ug/g flour)'
Free_FA_WW_flour='Free ferulic acid in whole wheat flour (ug/g flour)'
Free_FA_bran='Free ferulic acid in bran (ug/g bran)'
Free_FA_germ='Free ferulic acid in germ (ug/g germ)'
resistin_sample_ngml= 'Plasma resistin (ng/ml)'
fecal_bile_acid = 'Fecal bile acid (mg/24hr)'
TBARS_ug24hr = 'TBARS (ug/24hr)'
T0_MT_insulin = 'Insulin response to meal time 0'
T30_MT_insulin = ' Insulin response to meal time 30'
T60_MMT_insulin = 'Insulin response to meal time 60'
T120_MMT_insulin = 'Insulin response to meal time 120'
T180_MMT_insulin = 'Insulin response to meal time 180'
AUC_MMT_Insulin = ' Area under the curve for insulin response to meal
test'

;

;

*Concentration of total ferulic acid in wheat flours and fractions
(ug/g);
Tot_FA_ref_flour=67.05;
Tot_FA_WW_flour=581.50;
Tot_FA_bran=3031.28;
Tot_FA_germ=1006.54;

*Concentration of free ferulic acid in wheat flours and
fractions(ug/g);
Free_FA_ref_flour=3.04;
Free_FA_WW_flour=2.42;
Free_FA_bran=2.48;
Free_FA_germ=4.25;

*Calculation of the dietary concentration of total ferulic acid for
each diet (ug/g diet);
If Diet_Group=3 then Tot_FA_dietconc=153.7*Tot_FA_germ/1000;
If Diet_Group=4 then Tot_FA_dietconc=650*Tot_FA_WW_flour/1000;
If Diet_Group=5 then Tot_fa_dietconc=542.7*Tot_FA_ref_flour/1000;
If Diet_Group=6 then Tot_FA_dietconc=94.2*Tot_FA_bran/1000;
If Diet_Group=7 then Tot_fa_dietconc=16.2*Tot_FA_germ/1000;

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If Diet_Group=1 or Diet_group=2 then Tot_fa_dietconc=.;

*Calculation of the dietary concentration of free ferulic acid for each
diet (ug/g diet);
If Diet_Group=3 then Free_FA_dietconc=153.7*Free_FA_germ/1000;
If Diet_Group=4 then Free_FA_dietconc=650*Free_FA_WW_flour/1000;
If Diet_Group=5 then Free_FA_dietconc=542.7*Free_FA_ref_flour/1000;
If Diet_Group=6 then Free_FA_dietconc=94.2*Free_FA_bran/1000;
If Diet_Group=7 then Free_FA_dietconc=16.2*Free_FA_germ/1000;
If Diet_Group=1 or Diet_Group=2 then Free_FA_dietconc=.;

*Calculation of the daily total and free ferulic acid intake during
week 3 (ug);
Daily_Tot_FA_intake=Week3_FI*Tot_FA_dietconc;
Daily_free_FA_intake=Week3_FI*Free_FA_dietconc;

*Calculation of estimate of % absorption of ferulic acid from diet,
based on total and free ferulate in flours & fractions;
Tot_FA_absorpPC=(FA/Daily_Tot_FA_intake)*100;
Free_FA_absorpPC=(FA/Daily_free_FA_intake)*100;

*check rat 59 for high value;

output all_groups;
if Diet_Group = 2 or Diet_Group=3 or Diet_Group =4 or Diet_Group=5 or
Diet_Group=6 or Diet_Group=7
then output zdf_only;

Datalines;
1 1 1 128 149 184 211 224 253 125.0 13.35
14.80 17.70 15.28 98 143 148 165 171 142 157
190 0.19 104 127 140 98 104 95 92 103
35 . . . . 1.25 2.98
15.5 218 34 184 84.404 0.157 5.312 253
6.6115 1.5605 0.617 0.979 0.387 1.865 0.737 2.003 0.792
0.4053 7.02 74.41 68.20 450.89 27390 18930 . 6.752
44.64 2.082 13.765 100.13 34.49 28.51 12.20 67.11 56.52
23.08313842 3.5054 2.3282 0.422 0.4505 1.5517
0.5505 2.055 184.3515
2 1 1 128 151 195 224 234 252 124.5 15.30
15.65 17.15 15.65 81 133 178 146 147 187 152
200 0.2 90 147 108 75 90 87 70 92
47 35 34 30 39 75 1.35 2.95
17.7 232 41 191 82.328 0.146 5.600 252
8.9562 1.8718 0.743 0.812 0.322 1.077 0.428 1.579 0.627
0.4126 6.48 67.05 63.74 570.83 28373 16275 8108 6.678
59.81 2.107 18.872 101.55 33.52 32.45 13.29 84.27 84.88
21.5309377 3.2318 2.8651 0.5 0.532 0.7904
0.6471 35.316
3 1 1 111 137 171 195 206 223 112.4 15.25
13.75 14.10 14.37 117 134 169 148 177 178 164
179 0.179 112 134 110 102 103 98 100 97
53 39 42 40 59 86 0.8 2.63
21.4 204 44 160 78.431 0.139 4.839 223 7.456
1.526 0.684 0.940 0.421 0.938 0.420 1.252 0.561 0.3349 7.1
46.29 52.16 388.89 29370 18885 10095 5.011 37.36 1.963
14.634 101.43 35.94 22.76 9.77 64.32 58.74 20.91431001

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	2.815	1.9379		0.2857		0.3367		1.4871		0.6087	
	0.3368		127.932								
4	1	1	133	159	204	225	253	255	122.3		26.60
	16.95	20.50	21.35		91	125	152	160	176	154	156
	201	0.2	111	140	113	97	103	99	97		120
	46	49	36	46	89	77		1.62		3.27	
	19.5	242	47	195	80.579		0.145	5.906		255	
	7.6913		1.9174		0.752	1.065	0.418	1.071	0.420	1.449	0.568
	0.4145		6.77	87.36	73.61	566.15		27668	18840	11468	6.984
	53.71	2.068	15.904		101.06		31.57	35.18	14.08	74.34	87.56
	16.87433553	3.293	3.1008		0.4	0.5116		0.887	0.3965		
	0.4548		98.697								
5	1	1	131	159	199	212	228	242	111.3		16.85
	18.00	17.45	17.43		97	122	178	134	151	147	156
	185	0.18	116	138	119	102	100	94	93		85
	51	22		1.8		3.37	
	17.2	229	39	189	82.533		0.148	5.426		242	7.411
	1.7684		0.731	0.981	0.405	1.273	0.526	1.445	0.597	0.3827	
	6.92	86.56	82.98	614.93		26408	18698	1568	9.193	68.13	2.000
	14.823		99.68	29.48	38.18	15.04	79.39	71.02	18.1713	7997	
	3.2552		2.9015		0.3333		0.4	0.5716		0.4072	
	0.3154		76.6155								
6	2	3	192	226	280	315	341	360	168.0		21.25
	22.75	23.20	22.40		152	176	177	184	166	192	196
	282	0.282	136	232	224	189	144	144	147		143
	140	87	87	103	107	119		8.5		5.18	
	50.4	333	168	165	49.550		0.149	7.368		360	
	15.6131		2.1918		0.609	3.829	1.064	5.518	1.533	15.176	
	4.215	0.4841		6.7	120.55		97.28	1518.85		32783	30420
	19215	7.974	124.50		1.668	26.037		101.36		25.70	
	174.50		142.57		258.71		365.50		24.1936	101	
	3.8644		12.0962		2.3462		4.9593		3.3996		
	2.4483		1.6447		533.193						
7	2	3	184	221	275	310	334	352	168.0		24.97
	24.80	25.15	24.98		127	165	146	116	155	147	146
	278	0.278	111	204	211	183	140	137	139		137
	113	76	72	94	108	108		13.4		4.74	
	63.1	324	205	120	37.037		0.160	10.707			352
	12.1462		1.9533		0.555	4.288	1.218	5.779	1.642	15.594	
	4.430	0.3458		6.55	83.28	90.13	1094.74		25838	28665	17513
	7.135	86.66	1.879	22.823		100.82		38.33	152.62		
	156.77		246.01		347.19		22.87354	123	3.7473		6.296
	1.9257		3.6101		1.9444		0.5053		3.3504		
	355.5165										
8	2	3	182	216	265	296	324	340	158.0		21.25
	23.10	20.65	21.67		114	197	141	143	163	172	148
	271	0.271	161	240	213	207	145	154	158		135
	118	78	90	115	148	128		8.4		4.32	
	47.7	313	150	164	52.396		0.159	7.202		340	
	13.7201		2.1124		0.621	3.791	1.115	5.295	1.557	12.720	
	3.741	0.3995		6.6	97.32	95.51	1310.47		28343	31830	21188
	5.965	81.84	1.871	25.676		100.95		31.84	134.20		
	129.21		245.65		298.35		20.67342644		4.2732		
	10.1376		1.2373		1.3277		1.3603		0.8977		
	1.5704		220.578								
9	2	3	205	243	293	318	340	355	150.0		22.10
	23.30	24.70	23.37		117	150	134	152	164	163	181

	297	0.297	158	224	223	208	202	207	194		187
	143	96	91	112	165	155		17		5.59	
	52.6	337	177	160	47.478		0.155	7.538		355	
	18.1189		2.4789		0.698	4.090	1.152	5.734	1.615	12.848	
	3.619	0.4498		6.85	166.07		126.03		2283.53		28388
	36998	23873	8.957	162.29		1.714	31.061		101.18		26.53
	191.40		141.67		226.41		353.55		15.50793954		
	4.2904		12.7235		5.402	10.0446		5.917	9.4858		
	5.4591		1381.554								
10	2	3	193	235	282	315	342	353	160.0		24.10
	20.75	27.25	24.03		124	182	173	197	183	174	184
	294	0.294	113	165	294	158	160	126	140		138
	101	74	90	111	129	127		23.5		5.58	
	49.8	338	168	170	50.296		0.161	7.520		353	
	18.1068		2.6904		0.762	4.409	1.249	6.020	1.705	11.597	
	3.285	0.4305		6.73	158.62		115.27		2087.13		32303
	29348	19860	8.518	154.23		1.643	29.755		101.43		21.66
	197.65		127.90		161.71		221.74		19.90816912		
	5.0544		12.092		3.9801		2.0971		3.8738		
	4.5053		.		432.0945						
11	1	2	175	216	269	287	310	337	161.8		24.50
	22.75	15.35	20.87		164	235	229	159	204	219	200
	268	0.26	147	202	191	182	149	146	134		189
	140	81	82	124	156	158		4.8		4.95	
	51.7	307	159	149	48.534		0.161	6.720		337	
	16.5345		1.7864		0.530	3.639	1.080	4.503	1.336	9.832	2.918
	0.3356		7.01	245.63		216.97		3587.47		36653	28950
	23280	12.156		200.99		1.607	26.573		100.62		41.93
	47.76	12.84	185.52		182.65		22.21135445		3.5814		
	6.1267		0.4536		1.4617		7.7185		0.3336		
	5.1693		573.0825								
12	1	2	160	190	245	275	291	318	158.3		20.75
	22.05	.	31.58		108	149	155	136	273	161	196
	247	0.24	137	194	221	172	156	153	139		137
	112	97	77	130	137	156		5.4		4.58	
	63.2	284	180	105	36.972		0.157	9.072		318	
	12.6854		1.678	0.528	3.645	1.146	4.398	1.383	8.270	2.601	
	0.3391		7.31	125.09		107.74		1366.77		31928	29805
	21945	9.168	116.30		1.792	22.728		101.36		41.22	43.68
	11.52	123.72		182.52		20.08505209		2.6786		6.778	1.482
	2.237	2.668	0.8015		0.7082		278.736				
13	1	2	155	184	240	263	282	302	146.8		18.90
	21.20	20.00	20.03		117	171	113	156	194	199	182
	235	0.23	124	230	219	183	183	179	141		124
	133	73	77	118	133	142		3.8		4.15	
	62.3	275	172	104	37.818		0.155	9.031		302	
	11.553		1.6504		0.546	3.373	1.117	4.339	1.437	10.492	
	3.474	0.3192		6.9	137.81		111.03		1282.73		30900
	32573	20663	2.217	25.61	1.754	20.260		101.33		39.63	48.05
	13.24	130.16		157.15		22.38145864		2.0875		6.8051	
	3.445	5.4643		1.4537		1.6598		1.212	416.9685		
14	1	2	169	201	256	284	308	330	161.5		20.85
	21.95	20.40	21.07		139	196	124	157	155	195	210
	263	0.26	160	248	233	215	177	151	121		145
	131	60	75	100	145	129		5		4.19	
	53.2	300	160	140	46.667		0.165	7.380		330	
	14.1342		1.7737		0.537	4.072	1.234	4.464	1.353	9.577	2.902

	0.2552	6.97	163.18		138.88	1962.91	31208	32348		
	20048	11.587		163.78	1.833	25.903	101.25	48.24		
	64.31	19.97	164.99		183.41	23.18945354	2.8865			
	5.7858		1.388	5.0043		11.789	2.2418	6.9265		
	1043.757									
15	1	2	175	207	162	290	317	352	177.5	22.75
	23.35	19.95	22.02		139	193	152	152	155	166
	270	0.27	187	247	233	210	192	173	138	140
	130	59	88	113	138	132		4.1		4.74
	51.8	312	162	150	48.077		0.157	6.600		352
	15.642		1.994	0.566	4.227	1.201	5.315	1.510	13.647	3.877
	0.3435		6.6	199.9	161.30		2523.07	30248	34335	20528
	11.760		183.95		2.024	31.655		101.30		39.04
	509.51		5.88	149.37		165.67		20.63789071	3.382	
	4.6863		4.2735		29.801		13.9682	.		9.0356
	1167.6555									
16	2	2	178	209	267	288	315	327	149.0	21.95
	21.60	21.15	21.57		172	250	157	163	183	192
	265	0.265	152	195	201	185	160	164	165	137
	137	74	72	137	125	130		8.2		4.13
	54.6	308	168	140	45.455		0.147	6.984		327
	15.134		1.8494		0.566	3.653	1.117	4.873	1.490	15.191
	4.645	0.3373		6.93	203.35		158.84		2403.92	33233
	31268	20543	14.427		218.34		1.814	27.458		101.20
	31.19	48.92	15.09	155.34		136.08		23.52400995	3.1642	
	7.8335		5.332	10.875		3.2972		4.8635		5.5578
	1013.148									
17	2	2	186	218	284	297	319	335	149.0	20.75
	20.60	22.55	21.30		168	218	190	229	221	216
	272	0.272	144	277	254	224	186	162	165	160
	152	97	79	130	153	162		58.5		4.42
	54.5	314	171	143	45.541		0.156	7.240		335
	15.8483		1.9274		0.575	3.859	1.152	5.122	1.529	14.068
	4.199	0.3804		6.73	149.47		136.61		2165.11	38025
	35490	23678	12.882		204.16		1.819	28.828		101.16
	25.70	35.64	0.04	119.30		118.37		15.62272814	3.0837	
	4.9257		4.7369		2.4158		15.1639		6.1575	
	2.9553		1284.012							
18	2	2	193	224	254	267	314	342	149.0	21.95
	23.25	.	22.60		140	194	130	165	148	157
	262	0.262	142	225	196	152	108	128	121	176
	159	129	130	158	164	157		6.9		4.94
	52.4	311	163	148	47.588		0.155	7.108		342
	15.9663		2.0456		0.598	3.820	1.117	5.505	1.610	11.814
	3.454	0.3607		6.65	256.95		220.60		3522.10	28620
	26040	27338	13.237		211.35		1.762	28.139		101.08
	27.80	68.97	25.55	139.17		184.29		16.56016836	3.6798	
	6.3764		3.4606		4.6655		3.791	12.2871		0.9113
	1127.034									
19	2	2	168	205	285	285	312	331	163.0	24.20
	21.80	21.40	22.47		105	243	196	164	207	186
	265	0.265	158	252	258	235	220	215	211	152
	137	101	82	127	165	167		6.8		3.65
	54.6	306	167	139	45.425		0.153	6.837		331
	14.5412		1.8183		0.549	3.704	1.119	5.069	1.532	10.589
	3.199	0.3657		6.52	267.63		209.53		3046.79	33383
	40425	24173	18.028		262.15		2.272	33.033		101.29

	36.38	46.38	35.06	151.91	200.60	19.35335757	2.3507			
	4.7006		3.7102	5.7922	2.9553	2.4484				
	5.5296		675.1995							
20	2	2	196	233	284	317	346	365	169.0	24.50
	23.05	25.15	24.23		118	227	163	202	201	221
	297	0.297	129	229	209	187	161	168	168	136
	122	74	108	123	152	152		20.05	5.16	
	53.2	340	181	159	46.765	0.159	7.791		365	
	18.5417		4.1639		1.141	2.231	0.611	5.445	1.492	11.531
	3.159	0.3355		6.58	238.45		214.32		3973.78	35723
	32145	.	17.001		315.22		2.074	38.463		101.93
	25.56	34.75	41.02	150.77		153.02		21.38128946	2.2515	
	.	4.3413		9.5966		7.6407	.		3.1633	
	467.628									
21	1	4	164	194	245	273	285	298	134.3	20.25
	21.95	21.10	21.10		144	200	177	208	202	211
	247	0.24	113	213	194	153	118	110	116	120
	131	91	85	99	114	123		16		3.59
	56.2	280	158	123	43.929		0.153	5.981		298
	13.8018		2.092	0.702	3.853	1.293	4.352	1.461	8.349	2.802
	0.4743		6.17	120.08		118.26		1632.18		36503
	19253	11.608		160.21		1.952	26.939		100.48	29.33
	15.92	227.05		348.37		198.17		16.00255156	6.5087	
	9.7504		1.218	0.864	3.2061		1.0269		2.5994	
	328.0605									
22	1	4	157	185	240	268	286	306	149.3	19.00
	21.30	24.20	21.50		103	174	184	232	197	176
	245	0.24	116	187	157	132	167	108	.	117
	72	67	73	109	107	125		8		3.71
	50.4	283	142	140	49.470		0.144	5.743		306
	12.545		1.8591		0.608	3.231	1.056	3.209	1.049	7.698
	0.5232		6.01	109.35		94.67	1187.60		33953	17798
	6.879	86.29	1.590	19.944		101.39		38.71	11.37	196.59
	497.97		154.36		20.85052094	7.0962		7.4624		
	0.4969		0.7758		1.2261		0.7609		0.8155	
	156.021									
23	1	4	172	201	244	273	283	306	133.8	20.45
	22.25	23.80	21.35		99	146	132	114	170	125
	245	0.24	122	220	289	190	219	139	152	117
	100	64	95	112	124	121		29.5		3.79
	54.8	283	155	128	45.230		0.153	5.949		306
	15.048		2.0146		0.658	3.788	1.238	4.700	1.536	7.829
	0.5818		6.17	137.38		132.47		1993.34		25058
	19238	11.481		172.77		1.634	24.590		100.19	34.49
	22.57	279.28		339.44		237.08		15.21581969	6.9954	
	12.5215		4.604	10.6724		11.7723		1.4209		3.9063
	1121.4285									
24	1	4	182	213	265	293	316	336	153.6	20.40
	25.85	22.00	23.13		121	136	132	119	130	143
	275	0.27	115	187	155	118	105	108	131	113
	90	51	42	83	92	132		21		3.33
	52.7	312	164	147	47.115		0.141	6.756		336
	15.0468		2.237	0.666	3.662	1.090	4.733	1.409	8.088	2.407
	0.5536		5.66	140.84		112.90		1698.75		24923
	15195	11.754		176.86		1.857	27.938		101.10	36.77
	25.27	375.08		450.24		292.08		22.19009143	6.7331	

	11.0995	0.8121	2.388	6.6264	2.1184	1					
	539.1135										
25	1	4	187	209	253	283	311	325	138.1	23.45	
	24.75	23.90	24.03		156	131	116	109	124	137	165
	260	0.26	134	215	198	164	157	159	132		120
	79	67	69	127	132	129		18.5		3.78	
	52.3	311	163	148	47.588		0.152	6.964		325	
	17.0682		2.225	0.685	4.117	1.267	4.953	1.524	11.950		3.677
	0.5362		5.94	114.54		120.68		2059.84		23850	29430
	19283	9.883	168.69		1.802	30.752		101.39		23.02	14.37
	275.68		485.33		263.84		14.25898363	7.4601			
	11.0121		2.1935		8.4388		10.1971	1.4516			
	1.8893		888.711								
26	2	4	195	230	282	325	359	372	177.0	20.70	
	23.95	25.95	23.53		140	233	187	232	216	248	206
	293	0.293	123	223	210	161	110	147	148		121
	118	74	90	111	104	132		15		4	
	49.6	351	174	177	50.427		0.156	7.989		372	
	17.2865		2.3062		0.620	3.606	0.969	5.720	1.538	11.064	
	2.974	0.6004		6.66	133.48		130.10		2248.93		39533
	28178	19013	12.703		219.59		1.623	28.053		101.11	
	27.15	46.23	470.50		522.15		393.47		19.56380333		
	3.3955		12.2089		3.5634		5.0598		12.352		
	3.2939		1.5913		1006.458						
27	2	4	160	195	255	295	325	340	180.0	20.90	
	23.60	20.25	21.58		121	183	131	172	196	178	193
	262	0.262	138	198	204	180	133	149	128		135
	93	67	80	108	118	116		8.85		3.72	
	50.1	320	160	160	50.000		0.140	6.442		340	
	14.7619		2.3704		0.697	3.974	1.169	5.107	1.502	13.127	
	3.861	0.5631		6.68	96.03	100.50		1483.61		31440	28530
	18345	8.785	129.69		1.723	25.439		100.33		29.60	22.78
	256.19		549.23		198.27		21.62999809	2.9936			
	10.3587		2.0265		1.8558		4.1965		0.7505		
	2.2724		388.116								
28	2	4	226	252	295	332	359	364	138.0	23.40	
	23.00	24.65	23.68		120	178	117	133	130	162	203
	303	0.303	121	208	272	148	126	135	156		119
	108	83	73	100	107	124		27		4.18	
	50.7	354	179	174	49.153		0.153	7.618		364	
	18.1672		2.2281		0.612	4.229	1.162	6.040	1.659	14.293	
	3.927	0.571	6.76	137.26		115.64		2100.78		27473	29123
	18105	12.799		232.53		1.791	32.545		101.19		23.93
	21.40	361.30		521.40		357.41		20.04208915	5.9583		
	16.2505		2.6894		.	16.252		5.7815		3.0028	
	924.534										
29	2	4	188	223	270	305	330	353	165.0	20.40	
	21.55	26.00	22.65		113	171	130	201	226	183	188
	285	0.285	119	213	224	161	145	149	163		124
	113	71	66	105	136	150		11.5		4.07	
	51.5	326	168	158	48.466		0.158	7.407		353	
	15.6103		2.3973		0.679	3.752	1.063	4.911	1.391	11.123	
	3.151	0.4662		6.43	106.45		103.73		1619.29		33023
	29903	19973	8.392	130.99		1.589	24.798		100.66		31.51
	50.10	434.17		658.71		364.94		24.09795294	4.6911		
	10.7469		2.0107		3.0558		6.2158		3.761	5.6988	
	798.1695										

30	2	4	196	232	276	292	306	314	118.0		21.50
	25.30	27.65	24.82		144	186	140	154	153	167	178
	275	0.275	140	201	224	181	153	158	161		170
	156	81	98	119	155	205		55.5		3.99	
	49.2	296	146	150	50.676		0.157	7.055		314	
	18.3242		2.4717		0.787	3.553	1.131	4.931	1.570	11.280	
	3.592	0.3816		6.2	130.51		131.95		2417.85		29085
	31065	25073	14.357		263.09		1.446	26.505		101.24	
	17.77	25.05	377.65		484.50		332.11		14.93399656		
	4.0293		20.4231		5.904	16.9536		5.4591		6.3788	
	6.8262		1430.3415								
31	1	5	136	170	224	258	275	300	164.1		20.20
	21.40	22.70	20.80		151	197	190	196	219	201	206
	231	0.23	125	199	186	171	151	133	132		139
	108	68	97	117	121	116		9.8		3.88	
	50.7	271	138	134	49.446		0.160	6.374		300	
	13.5883		1.9948		0.665	3.263	1.088	3.750	1.250	7.317	2.439
	0.4126		6.88	117.1	98.14	1333.55		36038	27713	19538	8.841
	120.13		1.696	23.049		100.59		40.81	39.97	74.50	
	198.74		202.69		23.4233468	2.9337		8.2698		2.111	
	10.3992		4.0298		1.2786		3.237	698.808			
32	1	5	159	187	243	277	291	319	160.0		21.25
	22.05	23.40	22.23		117	131	155	200	170	212	201
	247	0.24	192	238	252	196	160	137	146		125
	116	81	111	126	140	121		6.4		3.63	
	53.7	290	156	134	46.207		0.146	6.092		319	
	12.544		1.8376		0.576	3.530	1.106	5.293	1.659	9.063	2.841
	0.465	6.8	107.19		94.42	1184.35		33000	31905	21540	7.647
	95.93	1.669	20.939		100.72		54.38	21.58	38.02	143.54	
	224.57		21.8924091		3.3182		4.8057		0.5627		
	2.9056		3.2594		0.7195		1.2025		321.5265		
33	1	5	183	215	265	290	303	318	135.0		20.45
	26.05	23.60	23.37		152	202	155	179	153	182	219
	260	0.26	133	233	189	187	138	125	114		93
	82	52	47	79	112	128		28.5		5.67	
	51.9	300	155	144	48.000		0.159	6.848		318	
	17.0641		2.3688		0.745	3.726	1.172	5.064	1.592	9.179	2.886
	0.474	6.57	151.53		161.03		2747.85		32378	27540	15758
	17.816		304.01		2.604	44.434		101.52		23.82	45.72
	64.64	199.14		227.99		12.04762917	4.3861		13.8252		
	5.0982		9.8751		1.1707		1.0766		3.0306		
	580.9215										
34	1	5	176	215	273	307	330	353	176.7		24.00
	26.50	25.25	25.25		132	174	112	133	160	174	205
	282	0.282	154	216	206	176	148	153	138		117
	103	41	71	106	123	153		9.4		4.33	
	63.3	322	204	118	36.646		0.159	10.681		353	
	16.2914		2.1317		0.604	3.867	1.095	5.447	1.543	10.657	
	3.019	0.4131		6.8	108.62		112.01		1824.87		28890
	29775	18780	9.405	153.22		1.679	27.357		101.52		37.89
	31.66	46.23	185.74		223.08		17.78864555		3.6029		
	9.6327		3.6044		6.793	4.479	1.4633		4.4792		
	681.585										
35	1	5	192	219	261	289	305	335	142.7		28.65
	26.05	25.05	26.58		144	196	153	175	147	159	189
	265	0.26	116	171	176	145	145	132	131		120
	96	62	71	100	113	121		7.8		4.06	

	49.6	300	149	151	50.333	0.160	7.120	335		
	14.9641		2.1642		0.646	3.387	1.011	5.536	1.653	11.056
	3.300	0.4848		6.89	117.87		122.15		1827.79	29948
	25965	17580	11.747		175.78		1.928	28.852		101.15
	40.01	28.56	47.15	204.25		173.55		20.0425	2605	3.9073
	7.8512		2.4203		12.1587		2.3733		4.3324	
	5.3642		928.734							
36	2	5	191	226	274	309	334	348	157.0	21.33
	23.75	22.25	22.44		131	194	115	125	167	164
	282	0.282	133	212	196	169	156	138	140	132
	109	91	89	104	115	117		5.3		4.19
	54.3	325	176	149	45.846		0.160	7.687		348
	13.7559		2.0398		0.586	3.991	1.147	4.376	1.257	14.398
	4.137	0.4319		7.08	115.01		95.92	1319.49		27450
	19148	10.357		142.47		1.800	24.762		101.32	35.41
	35.81	34.95	202.40		225.35		18.2628	6589	4.5971	
	6.5065		4.22	5.3984		3.1272	.		4.8345	
	272.16									
37	2	5	186	217	266	295	320	337	151.0	20.95
	20.30	24.30	21.85		146	175	116	142	166	195
	265	0.265	125	195	191	158	120	122	137	140
	107	71	86	107	118	129		15		3.83
	49.3	316	156	160	50.633		0.163	7.658		337
	14.6512		2.2483		0.667	3.605	1.070	4.960	1.472	11.403
	3.384	0.5094		7.03	110.34		111.16		1628.67	30675
	26100	19223	11.062		162.08		1.757	25.736		100.27
	38.00	37.68	62.11	221.68		214.77		22.0126	2675	4.1836
	9.8233		4.9711		7.4978		8.0948		5.0681	
	2.7453		1050.2115							
38	2	5	175	207	261	289	313	330	155.0	21.05
	23.20	22.95	22.40		130	191	126	180	230	199
	260	0.26	138	245	219	178	163	134	158	124
	120	83	82	124	154	136		5		3.9
	55.7	306	171	136	44.444		0.147	6.674		330
	13.1756		1.9671		0.596	3.372	1.022	4.039	1.224	12.260
	3.715	0.437	7.06	105.42		89.12	1174.21		32610	30638
	7.707	101.54		1.741	22.933		101.31		36.85	36.81
	188.18		250.47		18.2628	6589	3.7731		6.8763	
	6.6442		5.0404		10.255		9.7871		2.2435	
	1366.881									
39	2	5	189	220	261	293	311	329	140.0	21.40
	22.60	25.60	23.20		123	243	169	199	243	193
	270	0.27	110	169	155	138	132	135	138	100
	98	69	75	121	152	131		15		4.36
	54.1	302	163	139	46.026		0.162	6.886		329
	14.7276		1.9718		0.599	3.671	1.116	4.966	1.510	12.595
	3.828	0.3584		6.99	157.83		129.85		1912.35	36435
	25163	20423	13.284		195.64		1.882	27.712		101.41
	29.10	58.14	34.73	256.66		468.51		16.1584	0826	3.9256
	7.3129		1.978	16.0877		6.1786		10.6476		2.6485
	1508.649									
40	2	5	196	234	276	308	333	346	150.0	23.40
	21.65	25.02	23.36		135	193	156	170	159	195
	291	0.291	101	169	185	189	147	143	147	123
	118	85	85	126	139	171		7.5		3.56
	53.8	329	177	152	46.201		0.154	7.632		346
	14.1223		2.1216		0.613	3.752	1.084	5.352	1.547	11.709

	3.384	0.4023	6.93	155.66	141.38	1996.62	32363			
	28380	22320	12.636	178.45	1.869	26.393	100.97			
	37.68	41.83	34.95	197.37	240.31	16.63669409	3.6839			
	8.7708	1.6903	13.2606	4.8212	6.4298					
	6.3969	1217.8215								
41	1	6	187	228	282	316	331	342	155.2	23.70
	24.95	27.25	24.33	159	248	173	178	221	201	175
	286	0.28	134	181	170	166	153	135	125	162
	125	85	92	99	133	164	22	7.1		
	61.8	327	202	125	38.226	0.162	10.878			342
	18.505	2.4252	0.709	3.954	1.156	5.257	1.537	11.025		
	3.224	0.3454	6.7	134.63	110.82	2050.64				28860
	26940	21638	8.500	157.29	1.687	31.218	101.04			22.69
	61.08	299.24	336.67	357.83	15.74739528	7.149				
	12.3396	0.895	5.482	4.1319	3.2184	2.462	630.7845			
42	1	6	153	190	244	279	298	323	169.7	21.65
	23.00	24.20	22.95	125	162	142	161	221	193	177
	250	0.25	160	225	247	184	158	146	144	121
	118	64	84	100	113	125	6	4.29		
	50	291	145	146	50.172	0.160	7.044	323		
	13.6405	2.013	0.623	3.741	1.158	4.186	1.296	8.347	2.584	
	0.3685	6.48	94.63	72.76	992.47	32018	31283	18473	5.372	
	73.28	1.581	21.562	101.90	40.95	30.69	275.79			
	355.85	331.70	22.19009143	4.0514	8.1556					
	0.5255	7.0734	0.6456	0.8264	0.9456					
	327.0885									
43	1	6	186	221	278	310	332	333	147.2	22.55
	29.60	28.95	27.03	149	209	140	150	197	209	181
	280	0.28	115	179	185	150	130	124	120	114
	108	65	56	69	108	120	58	6.08		
	51.3	329	169	160	48.632	0.155	6.853	333		
	18.3465	2.4173	0.726	4.149	1.246	5.761	1.730	8.770	2.634	
	0.5175	7.12	153.35	142.68	2617.64	32648	25290			
	16148	13.016	238.80	2.009	36.858	101.49	25.61			
	67.81	490.12	398.06	422.99	13.74867106	6.8462				
	.	4.9812	10.8575	1.5396	5.8578	2.4906				
	895.911									
44	1	6	172	206	163	303	325	345	172.9	24.15
	26.45	23.20	24.60	114	173	131	151	160	189	196
	279	0.279	159	228	201	178	152	149	144	124
	101	64	60	92	124	146	13	5.57		
	64.4	313	202	111	35.463	0.158	9.534	345		
	14.4325	1.9644	0.569	4.362	1.264	6.075	1.761	9.978	2.892	
	0.3661	7.36	108.87	95.57	1379.27	30113	30060	18405		
	7.827	112.97	1.888	27.243	100.75	32.72	46.03			
	432.64	421.69	505.77	16.83180948	6.2804					
	10.4578	2.55	6.2509	4.3516	3.3982	4.1559				
	750.168									
45	1	6	166	192	235	264	296	322	156.1	23.85
	24.20	23.25	23.77	150	198	142	191	201	205	198
	240	0.24	151	213	212	143	157	143	136	130
	117	67	71	105	116	127	6.4	5		
	46.1	291	134	157	53.952	0.166	7.485	322		
	14.225	2.0515	0.637	2.986	0.927	4.501	1.398	8.717	2.707	
	0.3766	6.55	122.2	101.91	1449.67	34215	28613	18548		
	8.707	123.86	1.585	22.542	101.27	39.04	68.92			
	234.53	446.67	257.87	24.91175845	3.7326					

	7.4446	0.4194	2.0813	1.1843	1.1775					
	2.6997	273.6645								
46	2	6	192	231	283	320	352	368	176.0	23.15
	23.85	25.75	24.25		157	234	175	177	181	203
	292	0.292	148	244	230	192	159	128	154	143
	144	102	80	118	121	128		8.2		4.99
	51	347	177	170	48.991		0.156	7.545		368
	14.3895		2.1335		0.580	4.194	1.140	5.174	1.406	12.032
	3.270	0.4487		6.76	107.8	104.91		1509.67		33780
	20753	8.457	121.69		1.700	24.469		101.32		28.45
	250.29		500.43		306.44		20.04208915	5.0499		45.44
	9.7173		4.0479		12.1379		12.1243		4.0373	
	4.8206		1357.305							
47	2	6	152	182	230	266	290	302	150.0	17.75
	20.60	22.30	20.22		108	214	157	146	162	168
	235	0.235	153	276	245	187	167	165	146	143
	104	71	89	117	122	117		6.8		4.57
	54.6	280	153	127	45.357		0.159	6.896		302
	10.7227		1.6363		0.542	3.544	1.174	4.381	1.451	12.524
	4.147	0.3387		6.93	92.62	87.97	943.33		29603	33225
	6.035	64.72	1.672	17.933		101.05		45.44	39.66	251.41
	584.49		327.00		19.64032906	4.7678		7.2665		1.311
	3.1485		2.2365		2.24	1.357	389.8725			
48	2	6	194	224	267	297	318	334	140.0	23.10
	20.80	24.50	22.80		137	228	201	180	202	199
	278	0.278	120	184	192	223	200	165	162	147
	121	63	87	122	131	171		6.8		4.34
	57.9	312	181	131	41.987		0.149	6.895		334
	12.8906		2.0541		0.615	4.018	1.203	5.495	1.645	13.449
	4.027	0.3553		6.75	91.34	100.16		1291.18		34665
	21630	8.992	115.91		1.799	23.190		100.79		29.42
	265.94		624.32		390.88		10.70594988	3.9526		56.18
	6.8495		0.8703		9.8262		2.3488		7.191	4.1102
	968.3025									
49	2	6	196	230	276	305	328	348	152.0	21.75
	21.00	25.00	22.58		164	287	212	223	250	185
	288	0.288	142	168	167	140	122	126	122	128
	108	54	78	100	137	120		8.6		5.01
	53.5	321	172	149	46.417		0.151	7.116		348
	13.6027		2.1557		0.619	3.692	1.061	4.785	1.375	9.975
	0.3165		6.9	99.2	104.45		1420.86		39540	24533
	10.491		142.71		1.771	24.095		100.39		18900
	266.33		539.50		288.68		19.18117467	4.5533		56.14
	8.5464		3.2585		4.7735		9.3786		12.5523	
	2.5875		1444.8825							
50	2	6	198	238	269	310	340	354	156.0	25.00
	13.45	19.40	19.28		178	251	195	267	242	246
	294	0.294	143	193	236	249	226	190	193	149
	153	94	94	130	170	175		6.4		4.54
	63.2	326	206	120	36.810		0.167	11.429		354
	14.5118		1.9674		0.556	4.279	1.209	5.435	1.535	14.208
	4.014	0.3054		6.75	86.92	86.60	1256.72		41978	37868
	7.732	112.20		1.711	24.836		101.09		28.95	30.23
	184.77		484.71		221.05		20.36732351	3.8393		8.662
	3.4651		5.7276		7.6407		7.3589		4.3142	
	1138.596									

51	1	7	159	195	242	272	288	314	155.2	23.60
	23.00	21.58	22.73		127	185	190	180	280	198
	249	0.24	128	194	177	167	157	131	129	149
	112	68	78	109	132	120		5.7		5.18
	56.3	289	163	126	43.599		0.150	6.046		314
	14.4887		2.2266		0.709	3.444	1.097	3.737	1.190	10.055
	3.202	0.3494		6.94	148.36		125.40		1816.87	37013
	27338	19478	9.474	137.27		2.111	30.585		101.74	36.59
	41.40	17.50	97.24	143.84		20.91431001	4.0012			5.8123
	4.9067		3.5123		1.2577		0.677	3.4654		380.148
	1	7	142	176	234	270	290	324	181.9	21.65
	22.40	23.05	22.37		129	174	173	156	185	178
52	241	0.241	153	227	199	173	147	134	130	128
	101	68	101	131	142	115		7.4		4.48
	66.7	281	187	94	33.452		0.158	9.841		324
	13.2501		1.8464		0.570	3.571	1.102	4.906	1.514	10.788
	3.329	0.3071		6.66	107.09		101.95		1350.87	31890
	28560	20805	8.326	110.32		1.734	22.971		100.56	45.65
	103.87		35.11	199.88		303.49		18.25643206	3.2892	
	8.2189		0.6894		10.0089		0.814	2.8873		3.1557
	615.147									
	1	7	192	228	283	317	346	366	174.4	24.70
	25.95	24.65	25.10		144	181	215	177	207	180
	291	0.29	119	188	183	148	134	127	127	128
53	86	75	62	81	131	115		10.25		5.35
	64.1	334	214	120	35.928		0.154	10.359		366
	16.6927		2.1533		0.588	4.190	1.145	5.353	1.463	10.318
	2.819	0.4032		6.49	155.82		155.46		2595.09	33593
	25815	17573	10.632		177.48		1.911	31.902		101.18
	46.47	53.83	17.88	138.72		196.95		23.91239634	3.077	
	7.1268		2.9712		6.4573		2.9727		5.0512	
	5.8171		849.6435							
	1	7	180	217	270	293	320	334	154.2	21.35
	24.45	20.60	22.13		113	126	126	170	176	201
	272	0.27	158	224	195	177	157	131	133	134
	85	68	81	107	131	140		5.6		4.33
54	52.7	315	166	149	47.302		0.156	7.281		334
	11.6951		1.9605		0.587	4.191	1.255	5.551	1.662	13.460
	4.030	0.4183		6.9	101.95		99.10	1158.94		31508
	19545	7.834	91.62	1.789	20.919		100.68		47.27	50.21
	134.35		213.32		18.41591735	3.8665		6.8819		
	3.2284		5.4085		5.082	6.9092		4.8576		999.651
	1	7	173	198	241	273	301	319	145.9	21.25
	22.40	22.75	22.13		157	218	184	174	222	208
	250	0.25	139	224	140	152	131	133	130	138
	105	61	82	116	114	133		5		4.49
	50.5	294	148	146	49.660		0.176	7.645		319
	12.1531		1.9277		0.604	3.653	1.145	4.783	1.499	9.992
55	0.3766		6.37	81.44	88.50	1075.58		35708	25928	19043
	102.50		1.979	24.051		101.50		26.35	49.37	16.50
	135.78		183.41		16.80887698	3.4469		5.7554		3.575
	3.4236		1.6856		3.832	7.0558		673.779		
	2	7	185	222	272	297	316	344	159.0	23.15
	24.40	20.20	22.58		159	289	165	164	173	180
	279	0.279	145	225	225	179	161	164	169	136
	122	69	75	111	148	149		7.6		5.27
	53.8	312	168	144	46.154		0.157	7.472		344

	13.1861	1.9067	0.554	4.009	1.166	5.027	1.461	12.309		
	3.578	0.41	7.18	95.6	85.87	1132.30	32250	32175	21113	6.108
	80.54	1.577	20.796		100.57	34.61	92.57	22.25	182.24	
	279.96	14.321	17907	4.6778		10.6969	1.9447			
	5.8251	5.7826		5.3258		6.074	965.9085			
57	2	7	184	213	261	298	324	342	158.0	23.10
	27.15	24.65	24.97		126	209	155	139	185	162
	270	0.27	163	249	214	166	179	162	163	140
	125	74	77	111	127	138		6.5		4.64
	53.1	317	168	149	47.003		0.149	6.802		342
	13.0817	1.9279			0.564	3.440	1.006	4.622	1.351	13.274
	3.881	0.4154		7.11	89.06	94.94	1241.93		30368	32303
	9.396	122.92		1.927	25.203		101.34		39.45	70.57
	198.48		298.81		21.343	02659	4.5959		8.9865	
	2.4283		8.1406		1.3845		1.2232		4.4729	
	550.524									
58	2	7	200	236	278	308	329	347	147.0	25.60
	.	26.35	25.98		128	238	179	171	157	137
	298	0.298	112	218	186	163	141	140	158	148
	154	121	104	125	142	159		19		5.58
	54.1	321	173	147	45.794		0.159	7.569		347
	17.964		2.2392		0.645	4.017	1.158	5.262	1.516	13.039
	3.758	0.3814		6.62	204.23		144.85		2602.16	29483
	28455	24173	12.183		218.85		1.973	35.441		101.93
	29.74	98.36	30.32	169.13		219.84		15.144	44232	4.2017
	10.9418		0.8975		16.2811		1.2411		6.2621	
	2.3428		1003.755							
59	2	7	183	219	269	304	323	350	167.0	21.95
	39.60	24.35	28.63		155	231	171	188	194	165
	287	0.287	135	206	215	181	150	135	132	119
	116	63	69	100	132	140		6.25		4.76
	52	314	164	151	48.089		0.165	7.613		350
	14.749		2.2024		0.629	4.005	1.144	5.484	1.567	11.050
	3.157	0.3886		7.2	143.38		104.12		1535.74	32970
	28905	19260	9.474	139.73		1.712	25.246		101.43	31.19
	42.80	17.07	132.74		184.51		17.841	97436	3.941	6.6268
	4.6053		10.9859		8.7639		7.7188		7.5754	
	1483.422									
60	2	7	197	237	283	317	340	365	168.0	24.60
	37.55	24.10	28.75		142	203	182	151	153	182
	297	0.297	118	204	192	181	161	124	126	121
	103	79	88	111	120	1258		8.4		4.91
	53.7	330	177	153	46.364		0.164	8.018		365
	13.9027		2.2346		0.612	3.921	1.074	5.332	1.461	12.894
	3.533	0.4488		7.1	140.81		111.36		1548.15	30885
	27885	19350	12.680		176.29		2.069	28.761		100.96
	30.57	82.10	26.26	268.46		331.07		16.215	80256	4.606
	10.059		2.9693		8.6367		0.6136		8.6997	8.656
	1112.9145									
61	1	3	187	224	280	315	326	346	159.1	20.50
	24.30	24.05	22.95		181	245	181	173	180	216
	283	0.28	123	180	175	153	146	142	125	141
	130	87	85	79	97	141		29		4.5
	61.5	320	197	123	38.438		0.163	10.433		346
	17.0218		2.1772		0.629	4.110	1.188	5.508	1.592	10.330
	2.986	0.3712		6.49	140.32		125.27		2132.40	35085
	26670	18480	10.143		172.65		2.119	36.072		101.89

	29.15	531.71		252.13		339.07		432.75		13.92003061
	4.0369		16.7054		1.8194		1.8385		2.3184	
	2.3709		2.1556		393.696					
62	1	3	175	209	264	287	309	333	157.7	22.60
	24.30	21.35	22.75		136	180	169	166	232	208
	263	0.26	126	160	165	143	131	133	113	118
	114	81	85	114	124	128		7.5		4.5
	60.9	295	179	115	38.983		0.159	9.562		333
	12.0112		1.8762		0.563	3.704	1.112	5.185	1.557	11.326
	3.401	0.4459		7.14	91.46	96.85	1163.34		34703	24653
	4.541	54.55	1.889	22.693		101.17		39.18	375.93	19808
	142.69		258.46		35.14	19.04725464	3.301	10.1945		
	0.7126		2.7943		0.5	1.0367		1.47	223.32	
63	1	3	181	220	266	300	323	339	157.9	25.50
	25.45	22.45	24.47		142	154	153	166	191	195
	271	0.27	133	203	224	191	168	130	137	125
	110	71	86	87	115	126		9.2		4.2
	63.8	314	201	114	36.306		0.148	8.979		339
	11.0424		1.7954		0.530	3.938	1.162	5.597	1.651	12.716
	3.751	0.2929		6.76	95.12	74.14	818.67		30713	29813
	4.119	45.49	1.833	20.243		100.69		54.88	81.79	25.09
	247.69		443.94		16.77061412	3.7563		9.4278		4.563
	4.9714		2.2111		1.5371		1.0425		440.5875	
64	1	3	194	232	285	317	343	373	178.8	23.70
	25.55	24.60	24.62		119	129	129	119	130	142
	293	0.29	142	168	200	154	153	156	142	118
	112	63	69	89	113	119		12.5		5.27
	51.1	337	172	165	48.961		0.162	7.610		373
	17.0418		2.3746		0.637	4.479	1.201	5.571	1.494	11.937
	3.200	0.4407		6.69	90.74	93.23	1588.79		24750	28575
	7.889	134.45		1.579	26.902		101.10		37.74	290.71
	159.40		237.80		522.99		15.71838531	3.859	10.3967	
	5.482	4.8746		3.4302		9.8686		4.5216		1110.591
65	1	3	164	192	237	263	293	315	151.5	22.35
	25.70	22.35	23.47		136	152	140	119	130	142
	238	0.23	107	179	185	137	135	112	115	120
	68	67	66	128	100	99		6.4		5.01
	48	283	136	147	51.943		0.157	6.488		315
	11.2358		1.9365		0.615	3.249	1.031	4.665	1.481	9.360
	0.4405		6.7	65.94	71.35	801.65		25470	24300	16718
	46.65	1.593	17.903		100.13		40.16	60.87	105.49	
	257.76		345.56		18.76028315	3.7534		8.0267		
	0.6757		3.4893		1.2089		1.6502		.	218.721
66	2	1	160	180	206	221	236	242	82.0	15.00
	12.65	17.40	15.02		100	203	151	140	160	147
	206	0.206	100	150	109	105	99	101	97	81
	55	32	31	38	45	88		3.5		3.27
	20.7	228	47	181	79.386		0.145	6.165		242
	1.6187		0.669	0.950	0.393	1.139	0.471	1.394	0.576	0.3653
	7.2	86.83	78.07	513.86		26948	19028	8888	6.599	43.44
	13.840		101.00		27.00	40.91	23.81	149.60		101.08
	13.38435049		4.134	3.1907		0.5945		0.653	0.5907	
	0.7067		0.6266		116.289					
67	2	1	151	178	215	240	256	265	114.0	17.60
	14.60	19.40	17.20		96	153	176	143	175	144
	219	0.219	110	156	133	117	100	109	105	115
	61	44	32	25	37	78		3		2.98

	18.5	256	47	209	81.641		0.156	6.414		265
	8.8707		1.9356		0.730	1.170	0.442	1.288	0.486	2.185 0.824
	0.3828		6.57	107.48		96.19	853.25		27135	20723 8483
	7.658	67.93	1.885	16.725		100.40		23.43	45.73	24.07
	182.08		193.20		13.99655634	2.9266		5.1642		0.556
	1.3848		0.7253		1.0817	0.6846		167.9625		
68	2	1	158	187	211	226	238	247	89.0	18.05
	16.05	17.60	17.23		112	129	154	153	141	146 145
	212	0.212	96	121	111	99	99	100	105	103
	50	25	33	38	66	82		3		3.2
	19.6	230	45	185	80.435		0.146	6.091		247
	7.0619		1.7721		0.717	1.060	0.429	1.132	0.458	1.671 0.677
	0.4005		6.92	86.18	69.54	491.08		25980	18623	9645 7.184
	50.73	2.002	14.137		101.04		23.28	41.05	31.55	111.34
	103.97		11.85383585		3.4164		4.9709		0.4863	
	0.6528		0.6543		1.1868		72.3195			
69	2	1	152	182	208	216	232	240	88.0	18.60
	13.80	17.65	16.68		115	151	160	131	188	146 136
	201	0.201	89	110	124	105	103	106	94	83
	35	24	36	40	39	83		2.9		2.72
	19	226	43	183	80.973		0.149	6.048		240
	6.6562		1.6228		0.676	1.051	0.438	1.233	0.514	1.782 0.742
	0.3904		6.77	68.13	65.08	433.17		26948	18938	8213 8.180
	54.45	2.074	13.808		101.46		23.43	46.43	24.24	102.49
	149.72		14.03481921		2.9921		5.3026		0.5245	
	0.7603		0.5253		0.6595		1.0332		124.881	
70	2	1	166	193	215	228	236	244	78.0	18.60
	13.65	18.10	16.78		122	153	147	145	148	142 142
	213	0.213	95	129	122	104	83	103	101	102
	33	34	36	30	32	83		3		2.8
	20.4	230	47	183	79.565		0.153	6.364		244
	6.9172		1.7411		0.714	0.971	0.398	1.113	0.456	1.833 0.751
	0.4021		6.63	52.42	55.98	387.23		25958	18668	7935 5.787
	40.03	2.007	13.886		100.89		23.43	35.01	19.62	127.84
	114.42		12.54256744		3.0464		4.1008		0.5012	
	0.9109		0.7475		0.8305		0.7		.	

;

```
proc sort data = all_groups; by Diet_Group;
```

```
proc format;
  value dietfmt
    1='Lean Control'
    2='Obese Control'
    3='High Germ'
    4='Whole Wheat'
    5='Refined Wheat'
    6='Bran'
    7='Germ';
```

```
proc print data= all_groups; by Diet_Group; format Diet_Group dietfmt.;
```

```
proc means data=all_groups n mean stderr min max; by Diet_Group; format
Diet_Group dietfmt.;
var Tot_FA_absorpPC Free_FA_absorpPC Daily_Tot_FA_intake
Daily_free_FA_intake FA;
```

```

run;

proc glm data= all_groups;
title 'One Way Analysis of Variance in all rats';
format Diet_Group dietfmt.;
class Diet_Group Block_No;
model Initial_BW--AUC_MMT_Insulin Daily_Tot_FA_intake--
Free_FA_absorpPC=Diet_Group Block_No;
Means Diet_Group / Duncan;
LSMeans Diet_Group / STDERR pdiff;
run;

proc glm data= zdf_only;
title 'One Way Analysis of Variance in ZDF rats';
format Diet_Group dietfmt.;
class Diet_Group Block_No;
model Initial_BW--AUC_MMT_Insulin Daily_Tot_FA_intake--
Free_FA_absorpPC=Diet_Group Block_No;
Means Diet_Group / Duncan;
LSMeans Diet_Group / STDERR pdiff;
run;

proc corr data=all_groups;
title 'Correlation of Fecal Bile Acids and Liver Cholesterol in all
rats';
format Diet_Group dietfmt.;
var Liver_total_chol_conc_true resistin_sample_ngml;
with fecal_bile_acid Body_Fat_DXA;
run;

proc corr data=zdf_only;
title 'Correlation of Fecal Bile Acids and Liver Cholesterol in zdf
rats';
format Diet_Group dietfmt.;
var Liver_total_chol_conc_true resistin_sample_ngml;
with fecal_bile_acid Body_Fat_DXA;
run;
quit;

```